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Potential cytotoxic effect of hydroxypyruvate produced from D-serine by astroglial D-amino acid oxidase

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D-Amino acid oxidase (DAO) is a flavoenzyme that exists in the kidney, liver and brain of mammals. This enzyme catalyzes the oxidation of D-amino acids to the corresponding α-keto acid, hydrogen peroxide and ammonia. Recently D-serine, one of the substrates of DAO, has been found in the mammalian brain, and shown to be a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor in glutamate neurotransmission. In this study, we investigated the metabolism of extracellular D-serine and the effects of D-serine metabolites to study the pathophysiological role of DAO. Treatment with a high dose of D-serine induced the cell death in dose-dependent manner in DAO-expressing cells. Moreover, overexpression of DAO in astroglial cells induced the enhanced cytotoxicity. The treatment with 1 mM beta-hydroxypyruvate (HPA), uniquely produced from the D-serine metabolism by DAO activity, also induced cell death, comprising apoptosis, in the astroglial cell, but not in the other cells derived from liver and kidney. Taken together, we consider that high dose of extracellular D-serine induced cell death by the production of not only hydrogen peroxide but also HPA as a result of DAO catalytic activity in astroglial cell. Furthermore, this cytotoxicity of HPA is observed uniquely in astroglial cells expressing DAO.

Keywords: D-Amino acid oxidase/cytotoxicity/β-hydroxypyruvate/oxidative stress/D-serine.

Abbreviations: ALS, amyotrophic lateral sclerosis; cDNA, complementary DNA; DAO, D-amino acid oxidase; FAD, flavin adenine dinucleotide; FBS, fetal bovine serum; GRHPR, glyoxylate reductase/hydroxypyruvate reductase; HPA, β-hydroxypyruvate; L-LDH, L-lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PAGE, polyacrylamide gel electrophoresis; PI, propidium iodide; NMDA, *N*-methyl-D-aspartate; ROS, reactive oxygen species; RT–PCR, reverse transcriptase PCR; PBS, phosphate-buffered saline.

D-Amino acid oxidase (DAO) is a flavoenzyme with flavin adenine dinucleotide (FAD) as its cofactor. This flavoenzyme catalyzes the oxidative deamination of neutral and basic D-amino acids to the corresponding imino acids and hydrogen peroxide (H_2O_2) with concomitant reduction of FAD (*1*). Subsequently, the imino acid is nonenzymatically hydrolyzed to the corresponding α-keto acid and ammonia (Fig. 1). DAO was reported to be intracellularly localized in the peroxisomes of the various species (*2*). In mammals, its existence was also reported in the kidney, liver and brain (*3–7*). Histochemical studies in the rat brain revealed that DAO activity was located to the some types of astrocytes in lower brain stem and cerebellum (*8*). We also reported the gene expression of DAO in type-1 astrocytes from rat cerebral cortex as well as the cerebellum (*9*).

Substantial amounts of free D-serine, one of the substrates for DAO, was first found in the mammalian brain in 1992 (*10*) although the existence of *in vivo* substrate for DAO was not known for many years. D-Serine is thought to be a gliotransmitter (*11*) and endogenous co-agonist for the glycine site of the *N*-methyl-D-aspartate (NMDA) receptor which is a ligand-gated Ca^{2+} channel. This NMDA receptor co-agonist can modulate glutamatergic neurotransmission through NMDA receptor (*12*, *13*). It has been reported that glia-derived D-serine controls NMDA receptor activity and synaptic memory (*11*). Furthermore, hypofunction of the NMDA receptor has been implicated in the pathology of schizophrenia, particularly regarding the negative and cognitive symptoms of the disease (*14*). Recent study found a significant decrease in cerebrospinal fluid D-serine levels in schizophrenia patients (*15*), and the increase in the plasma D-serine levels of drug-naïve patients was correlated with improvements in positive symptoms (*16*), even though there is still some controversy on the decreased concentration of D-serine in schizophrenia patients. In addition, the protein levels of hippocampal DAO in schizophrenia patients were significantly higher than in healthy control subjects.

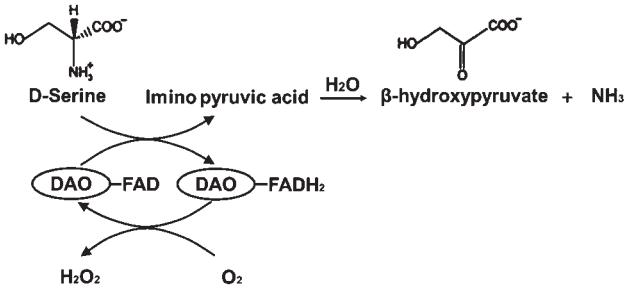

Fig. 1 General D-serine metabolic pathway in mammals. D-Amino acid oxidase (DAO) catalyzes the oxidative deamination of D-serine to the imino pyruvic acid and hydrogen peroxide (H_2O_2) with concomitant reduction of FAD. Subsequently, the imino pyruvic acid is nonenzymatically hydrolyzed to the β-hydroxypyruvate and ammonia.

In the previous report, we also reported that higher level of DAO expression was observed in schizophrenic choroid plexus epithelial cells than that in non-schizophrenic cases (*17*), and DAO activity was inhibited by addition of antipsychotic drugs such as chlorpromazine (*18*) and risperidone (*19*). Moreover, genetic linkage study implicated that DAO, together with its activator, G72, plays a key role in the pathophysiology of schizophrenia (*20*). However, the molecular mechanism through which DAO activity is modulated by the G72 gene product is still subject of dispute, because it was recently reported that pLG72, protein that encoded by G72 gene, acts as a negative effector of human DAO (*21*). Several clinical trials investigated whether administration of D-serine is beneficial or not for schizophrenic patients, and indicated that D-serine greatly improved symptoms in the patients (*14*).

On the contrary, massive stimulation of the NMDA receptors is a major cause in the neuronal degeneration associated with a variety of neurological disorders such as ischemia, epilepsy and trauma (*22–24*). These reports also indicated excitatory amino acids including D-serine may play a major role in pathophysiological conditions related with NMDA receptor-mediated neurotoxicity. Moreover, application of DAO markedly inhibited neuronal damage by NMDA or oxygen-glucose deprivation in rat (*25*). In addition, D-serine application to rats resulted in gene expression changes related in neurodegenerative disorders and neuronal dysfunction (*26*). Furthermore, high dose of intraperitoneally injected D-serine damaged rat proximal straight tubules, leading to acute necrosis of the proximal straight tubules which was accompanied by proteinuria, glucosuria and aminoaciduria (*27*, *28*).

In previous report, we showed treatment with a high dose of D-serine induced apoptosis followed by the production of H_2O_2 as a result of DAO catalytic activity in astroglial cells, suggesting astroglial DAO is involved in regulation of the extracellular level of D-serine (*2*). In the metabolism of D-serine involving DAO, β-hydroxypyruvate (HPA) as well as H_2O_2 is generated from the oxidative deamination of D-serine. HPA has been implicated as an intermediate in the synthesis of glucose from serine (*29*), and is involved in the oxalate metabolism (*30*, *31*). But the physiological role of HPA in the mammalian system is still not completely clear.

To investigate the potential role of DAO in D-serine metabolism and the effect of its metabolites, we examined the effect of D-serine, H_2O_2 and HPA on cell viability. Here, we show that treatment with a high dose of D-serine in DAO-expressing cells derived from brain and kidney induced cell death by the production of not only H_2O_2 but also HPA. Furthermore, HPA also induced decrease in cell viability, comprising apoptosis, uniquely in astroglial cells expressing DAO.

Materials and Methods

Reagents and antibody

D-Serine, H_2O_2, and catalase (*Aspergillus niger*) were purchased from Wako (Osaka, Japan). Sodium benzoate, Na-β-hydroxypyruvate (Na-HPA), Li-β-hydroxypyruvate (Li-HPA) and Na-pyruvate were obtained from Sigma (St Louis, MO, USA). G418 and penicillin–streptomycin–glutamine solution were purchased from Gibco BRL Life Technologies, Inc. (Grand Island, NY, USA). Edaravone was a gift from Mitsubishi Tanabe Pharma Corporation (Tokyo, Japan). To confirm the DAO expression in protein level, the rabbit polyclonal anti-porcine DAO antiserum (*7*) was used. Rabbit polyclonal antibody raised against caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal anti-β-actin antibody (Sigma) were used for Western blot analysis. To confirm the cytotoxic effect, CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit were purchased from Promega (Madison, WI, USA).

Cell culture

Rat glioma C6 cells were cultured in DMEM/F-12 (Invitrogen Co., Carlsbad, CA, USA) containing 15% horse serum and 2.5% fetal bovine serum (FBS). C6 cells were transfected with a plasmid encoding mouse DAO (*2*). Resistant clones were screened using G418. 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. C6/DAO cells were maintained under the same condition as C6. H4TG cells are a derivative of the H4-II-E-C3 rat hepatoma cell line. This cell line was cultured in Dulbecco's modified Eagle's medium with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and supplemented with 10% FBS. NRK-49F cells that are rat kidney cell line were cultured in Dulbecco's modified Eagle's medium containing 5% FBS. LLC-PK_1 cells, pig renal epithelial cells, were cultured in Medium 199 containing 5% FBS. This cell line has been described to undergo morphological changes during differentiation and maturation from subconfluent culture to a confluent epithelial layer. One characteristic morphological change is known as the formation of dome-like structure. The level of DAO expression was increased after dome-like structure formation (*19*). Therefore, LLC-PK_1 cell line after dome-like structure formation was used in this study. NIH 3T3 cells, mouse embryonic fibroblast cell line, were cultured in Dulbecco's modified Eagle's medium with 10% FBS. Rat primary type-1 astrocytes were prepared as described previously (*9*). Briefly, mixed glial cultures were prepared from Sprague–Dawley rat cerebellum on postnatal days 1–2 in poly-D-lysine–coated culture flasks containing Dulbecco's modified Eagle's medium supplemented with 10% FBS. After 10–14 days of culture, astrocytes were isolated by shaking to dislodge microglia and subsequently purified by cytosine arabinoside treatment. The purified type-1 astrocytes were replated onto multi-well plates for assays. All cells were cultured in the medium containing 1% penicillin–streptomycin–glutamine solution at 37°C under a humidified atmosphere containing 5% CO_2.

RNA isolation and reverse transcriptase (RT)–PCR

Total RNA was isolated from C6, C6/DAO, H4TG, NRK-49F and LLC-PK_1 cells with TRIzol reagent (Invitrogen Co.) according to the manufacturer's instruction. Concentration of total RNA was spectrophotometrically determined by measuring the optical density at 260 nm. Total RNA (1 μg) was reverse-transcribed using a

Table I. The primer sequences of DAO, β-actin and GRHPR.

Primer	Sequence
Forward primer of DAO	5′-TCAGGCTACAACCTCTTCCG-3′
Reverse primer of DAO	5′-CACTCCCCTCTCAGTTAACC-3′
Forward primer of β-actin	5′-CGACAACGGCTCCGGCATGT-3′
Reverse primer of β-actin	5′-CCAGCCAGGTCCAGACGCAG-3′
Forward primer of GRHPR	5′-CCAGCCCAGGCCTCAGGAAG-3′
Reverse primer of GRHPR	5′-AGGTGGCACTGCCGATGTGG-3′

SuperScript III First-Strand Synthesis System for RT–PCR kit (Invitrogen Co.) according to the supplier's instruction. The 2 μl of cDNA was amplified in Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using primers listed in Table I. The primer sequences used for the detection of DAO mRNA were identical to pig, rat and mouse DAO cDNAs. The primer sequences of glyoxylate reductase/hydroxypyruvate reductase (GRHPR) and β-actin mRNA were also designed by using the common sequences among species examined. After activation step of Taq enzyme (for 10 min at 96°C), PCR reactions were performed with following steps for 25 cycles (β-actin), 32 (DAO) or 28 (GRHPR): denaturation for 30 s at 95°C, annealing for 45 s (β-actin) or 120 s (DAO, GRHPR) at 60°C and extension for 45 s (β-actin), 150 s (DAO, GRHPR) at 72°C.

Quantitative real-time PCR assay

Quantitative real-time PCR was performed according to the manufacturer's protocol, using SYBR Premix Ex Taq II Kit (Takara Bio Inc., Shiga, Japan) and the 7500 Real-time PCR System (Applied Biosystems). The same sets of primers were used as those for RT–PCR. DAO gene expression was normalized to β-actin expression. Relative gene expression was calculated with the $2^{-\Delta\Delta C}{}_T$ method (*32*).

Western blot analysis

For western blot analysis, cells were collected by scraping in phosphate-buffered saline (PBS) and then treated with a lysis buffer (20 mM HEPES; pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany). Cells were disrupted by sonication, extracted at 4°C for 30 min, and then centrifuged at 14,000*g* for 20 min. Protein samples resuspended in denaturing sample buffer were electrophoresed on 12.5% (for detection of DAO and β-actin) or 15% (for detection of caspase-3) polyacrylamide gel containing 0.1% SDS. Proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA, USA). The membranes were incubated with rabbit polyclonal anti-porcine DAO antibody (1 : 4000), monoclonal anti-β-actin antibody (1 : 5000) and rabbit polyclonal anti-caspase-3 antibody (1 : 200). Detection of each protein was carried out with an ECL Plus (for detection of DAO) or ECL Advance (for detection of caspase-3) Western blotting detection system (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

Cell viability assay

The cell viability was determined by means of the MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay. In brief, cells (1×10^4) were seeded in 96-well plates and incubated for 24 h (C6, C6/DAO and NRK-49 F) or 72 h (H4TG, LLC-PK_1). Then cells were treated with different concentrations of several reagents including D-serine, H_2O_2 and HPA. Sodium benzoate, catalase and edaravone were added 30 min prior to D-serine treatment. In order to exclude a direct cytotoxic effect of sodium benzoate, catalase and edaravone, but to obtain maximal inhibition, 20 mM sodium benzoate, 0.75 unit/well catalase and 0.25 mM edaravone were used in the assay. At 22 h after incubation, 20 μl (1/5 cell culture volume) of CellTiter 96 AQ_{ueous} One Solution reagent added to each well, followed by further incubation for 2 h. The absorbance (490 nm) was measured using Infinite 200 Microplate Reader (Tecan, San Jose, CA, USA).

Determination of pyruvate and β-hydroxypyruvate release

The concentration of NADH oxidizing compounds (pyruvate, HPA and glyoxalate) was measured with an F-kit pyruvate (J.K international, Tokyo, Japan). In brief, C6, C6/DAO and LLC-PK1 cells (1×10^6) were seeded in 100 mm dishes and incubated for 48 h (C6, C6/DAO) or 96 h (LLC-PK_1). The cells were washed once with PBS and then incubated for 6 h with DMEM (no pyruvate) containing 1 % FBS and several concentrations of D-serine. After centrifugation at 10,000*g* for 10 min, the supernatant was collected for the assay. Samples were incubated with assay buffer containing NADH for 3 min at 25°C. Then, they were incubated with L-lactate dehydrogenase (L-LDH) for 5 min at 25°C. The absorbance was measured at 340 nm. In each experiment, HPA at known concentrations was determined in parallel as standards.

Flow cytometric analysis

To examine whether or not cell death was due to apoptosis after Na–HPA treatment, Annexin V assay was performed using ApoAlert Annexin V kit (Clontech, Mountain View, CA, USA) under the manufacturer's instruction. C6 cells (5×10^5 cells/well) were seeded in 6-well tissue culture plates. After 24 h incubation, the cells were treated with or without 1 mM Na–HPA and H_2O_2 for 6, 12, 18 or 24 h. Cells were harvested using 0.25% trypsin/EDTA solution. Then the cells were resuspended in 1× binding buffer and incubated with FITC-conjugated annexin V and propidium iodide (PI) for 15 min at room temperature in a light-protected area. Thereafter, the samples were analyzed with Coulter Epix XL Flow Cytometer (Beckman Coulter, Miami, FL, USA).

Statistics

All data were expressed as the means ±SE for three or more independent experiments. Statistical comparisons between different treatments were made using student's *t*-test. Differences were considered significant if $P < 0.05$.

Results

Expression of DAO in several cell lines

In search of the pathophysiological role(s) of DAO, we considered it important to analyze the expression profile of DAO. Therefore, we confirmed the DAO expression using RT–PCR and quantitative real-time PCR in C6, rat astroglioma, C6/DAO, DAO-overexpressing C6 cells, H4TG, rat hepatoma cells, NRK-49F, rat kidney cells, and LLC-PK_1, pig renal epithelial cells. As the result, we could observe the RT–PCR products of predicted size as a single band (Fig. 2A). Real-time PCR showed that the level of DAO mRNA was higher in C6/DAO (124-fold) and LLC-PK_1 (154-fold) than that in C6. However, the level of DAO mRNA in H4TG and NRK-49F was not significantly different from that in C6 (Fig. 2B). Furthermore, we also confirmed the DAO expression at protein level. A strong band at approximately 38.5 or 39 kDa was detected in C6/DAO and LLC-PK_1, respectively (Fig. 2C, lane 2 and 5). It has been reported that analysis of the nucleotide sequence of porcine DAO revealed a complete 3211 nucleotide sequence encoding a protein consisted of 347 amino acids, whereas that of mouse revealed a 1647 nucleotide encoding 345 amino acids (*3*, *6*). On the other hand, DAO could not be detected in C6, H4TG and NRK-49F.

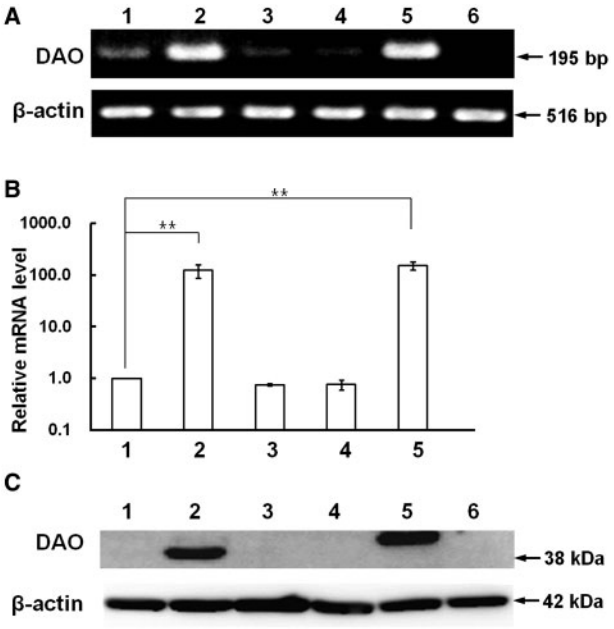

Fig. 2 Gene and protein expression of DAO in C6, C6/DAO, H4TG, NRK-49, LLC-PK$_1$ and NIH 3T3 cells. Gene and protein expression of DAO was measured in C6 (lane 1), C6/DAO (lane 2), H4TG (lane 3), NRK-49F (lane 4), LLC-PK$_1$ (lane 5) and NIH 3T3 (lane 6) cells using several methods. (A) Total RNA was extracted from cultured C6, C6/DAO, H4TG, NRK-49F, LLC-PK$_1$ and NIH 3T3 cells. Extracted total RNA was submitted to reverse transcriptase-PCR analysis, followed by electrophoresis on 2% agarose gel. (B) The level of DAO mRNA was measured by quantitative real-time PCR assay. Relative mRNA level of DAO was normalized by β-actin mRNA, and data are shown as the relative DAO mRNA level compared to C6 cells. Results are representative the mean ± SE of values obtained from three independent experiments. $^{**}P<0.01$, compared with C6 cells. (C) Western blot analysis was carried out to detect DAO protein expression with anti-pig DAO antibody. Protein samples were separated by electrophoresis on 12.5% polyacrylamide gels. A specific signal for DAO was detected at 38.5 or 39 kDa (upper panel). Blots were re-probed with anti-β-actin as a loading control (lower panel).

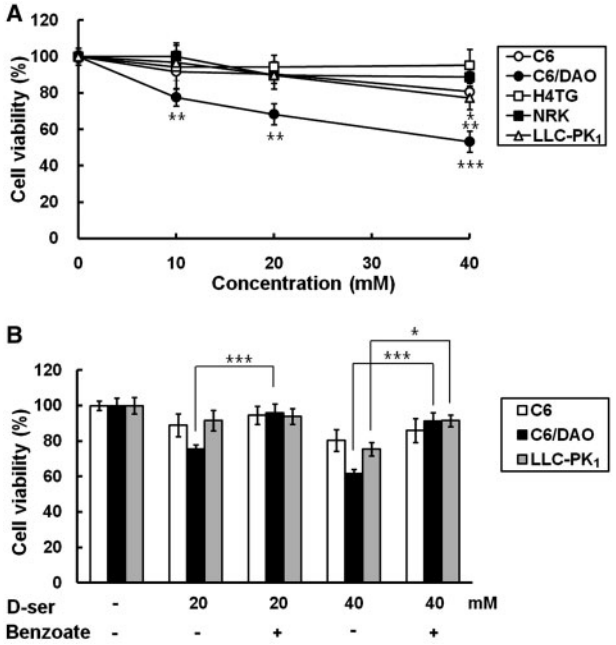

Fig. 3 Effects of D-serine on cell viability of C6, C6/DAO, H4TG, NRK-49 and LLC-PK$_1$ cells and the cell viability exposed to D-serine with or without DAO inhibitor sodium benzoate. (A) The cell viability was examined by means of the MTS assay after treatment with the indicated concentrations of D-serine in C6 (open circles), C6/DAO (closed circles), H4TG (open squares), NRK-49F (closed squares) and LLC-PK$_1$ (open triangles) cells for 22 h. Results represent the mean ± SE of values obtained from six independent experiments. (B) C6, C6/DAO and LLC-PK$_1$ cells were pretreated with 20 mM sodium benzoate for 30 min before D-serine treatment. The cells were then treated with 20 or 40 mM D-serine for 22 h. Cell viability was also measured by means of the MTS assay. Results represent the mean ± SE of values obtained from four independent experiments. The viability of untreated cells was normalized to 100%, and the data are means ± SE of the percentage of controls. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with control.

The effect of high dose of D-serine treatment

In order to confirm the metabolism of extracellular D-serine in these cell lines, we examined the cell viability of these cells 22 h after the treatment with 10, 20 and 40 mM D-serine. As shown in Fig. 3A, the cell viability of C6, C6/DAO and LLC-PK$_1$ cells was significantly decreased in a dose-dependent manner, whereas that of H4TG and NRK-49F cells was not significant. Moreover, C6/DAO cells showed higher sensitivity to D-serine treatment even though the DAO expression level of C6/DAO cells was lower than that of LLC-PK$_1$. These results indicated that the cytotoxic effect of D-serine on astroglial cells is greater than that on other cells. Furthermore, cytotoxic effect of D-serine on C6/DAO cells was greater than that on C6 cells, indicating that intracellular DAO is involved in the cytotoxicity of D-serine in astroglial cells. 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 20 and 40 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 as reported previously (*2*). As shown in Fig. 3B, the cell viability of C6/DAO and LLC-PK$_1$ cells was significantly recovered. Furthermore, the recovery of C6 cells pretreated with sodium benzoate was also observed. These results indicated that DAO activity was involved in the cytotoxicity induced by D-serine.

Cell viability in DAO-expressing cells treated with H_2O_2

In order to investigate why astroglial cells showed higher sensitivity to D-serine treatment, we examined the effects of D-serine metabolites produced by DAO activity on DAO-expressing cells including C6/DAO and LLC-PK$_1$.

At first, we evaluated the cell viability of DAO-expressing cells 22 h after treatment with 0.25, 0.5, 0.75 and 1 mM H_2O_2, common product of D-amino acids metabolism. As shown in Fig. 4A, the cell viability of DAO-expressing cells was decreased in dose-dependent manner after treatment with H_2O_2, indicating that H_2O_2 as a product of D-serine metabolism can induce cell death. Moreover, no significant difference was detected between C6 and

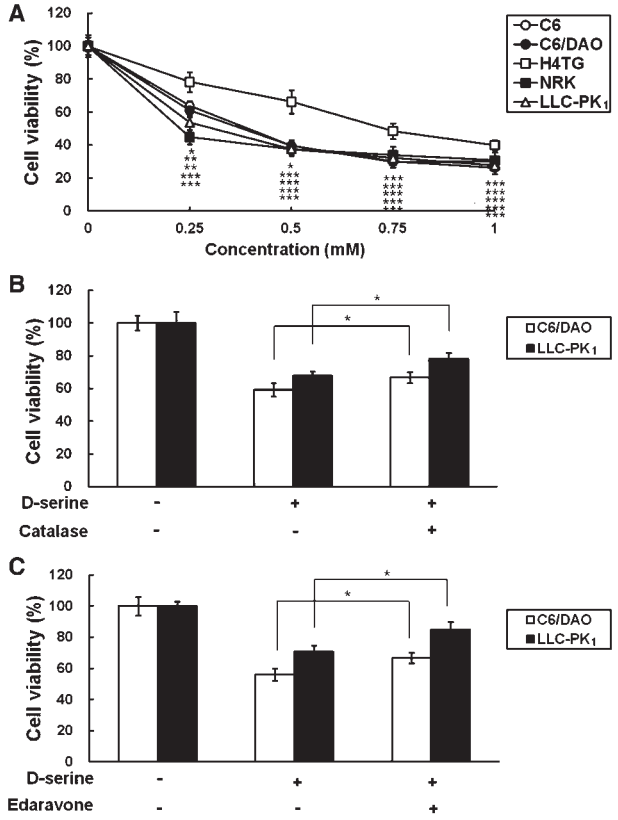

Fig. 4 Effects of H_2O_2 on cell viability and protective effects of catalase and edaravone on D-serine-induced cytotoxicity. (A) The MTS assay was performed 22 h after treatment with the indicated concentrations of H_2O_2 in C6 (open circles), C6/DAO (closed circles), H4TG (open squares), NRK-49F (closed squares) and LLC-PK_1 (open triangles) cells. Results represent the mean ± SE of values obtained from six independent experiments. (B, C) C6/DAO and LLC-PK_1 cells were pretreated with 0.75 unit/well catalase (B) and 0.25 mM edaravone (C) for 30 min before D-serine treatment. The cells were then treated with 40 mM D-serine for 22 h. Cell viability was also measured by means of the MTS assay. Results represent the mean ± SE of values obtained from four independent experiments. The viability of untreated cells was normalized to 100%, and the data are means ± SE of the percentage of controls. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with control.

C6/DAO, indicating that the cytotoxicity of H_2O_2 is not dependent on the intracellular level of DAO.

Then, in order to confirm whether H_2O_2 is involved in D-serine-induced cell death, C6/DAO and LLC-PK_1 cells were pretreated, before exposure to 40 mM D-serine, and incubated with catalase (0.75 unit/well), common enzyme that plays a role in detoxifying H_2O_2. As shown in Fig. 4B, pretreatment and incubation with catalase at a concentration of 0.75 unit/well significantly prevented the cell death induced by D-serine (40 mM) in C6/DAO and LLC-PK_1 cells. These results indicated that catalase decomposed H_2O_2 released from the cell, leading to the decrease in cytosolic H_2O_2 level.

In addition, the effect of edaravone, a free radical scavenger, was also examined. Edaravone was added to C6/DAO and LLC-PK_1 cells 30 min before exposure to 20 and 40 mM D-serine. As shown in Fig. 4C, the cell viability of C6/DAO pretreated with 0.25 mM edaravone was significantly recovered to approximately 67%, whereas that of LLC-PK_1 was approximately 90%. These results indicated that H_2O_2 is involved in D-serine-induced cell death. Moreover, these results suggested that the cytotoxicity of C6/DAO induced by D-serine was caused not only by H_2O_2 but also by other factors, because the recovery of C6/DAO cells by edaravone was reached only to 67% even though that of LLC-PK_1 cells was 90%.

Cell viability in DAO-expressing cells treated with HPA

We next examined the cytotoxicity of HPA, another product of D-serine metabolism, 22 h after treatment with 0.25, 0.5, 0.75 and 1 mM Na-HPA. As shown in Fig. 5A, the cell viability of H4TG, NRK-49F and LLC-PK_1 cells was not affected by the addition of Na-HPA. However, C6 and C6/DAO cells were sensitive to Na-HPA treatment in dose-dependent manner at concentrations higher than 0.5 mM. Another type of salt of HPA, Li-HPA, was examined for the potency of cytotoxicity. Sodium-pyruvate was used as control based on its structural similarity to HPA. Although Na-pyruvate did not show cytotoxic effect, we could detect the cytotoxicity of both types of HPA on C6 (Fig. 5B) and C6/DAO cells (Fig. 5C). In addition, we observed little difference between C6 and C6/DAO, indicating that the cytotoxicity of HPA is not dependent on the intracellular level of DAO.

Furthermore, we evaluated the cell viability of rat primary astrocytes to confirm the effect of HPA on astroglial cells. As shown in Fig. 6, rat primary type-1 astrocytes also exhibited the decrease in viability on Na-HPA treatment in dose-dependent manner at concentrations higher than 0.5 mM. Taken together, HPA is considered to cause cell death in astroglial cells at lower concentration than that of D-serine, and be involved in the cell death of C6/DAO induced by D-serine.

Estimation of released HPA in C6, C6/DAO and LLC-PK_1 cells treated with D-serine

We next examined the correlation between cell viability and the production of HPA during the metabolism of D-serine. The F-kit pyruvate was used for the detection of HPA on the basis of NADH oxidation using L-LDH enzyme. The catalytic degradation of D-serine by DAO generates HPA. Therefore, we speculated that the increased value in C6/DAO and LLC-PK_1 cells treated with D-serine corresponded to the amount of HPA release. As shown in Table II, we detected an increased value in C6/DAO cells treated with 20 and 40 mM D-serine for 6 h and the increased value in C6/DAO cells treated with 40 mM D-serine was significantly higher than that in C6 cells. LLC-PK_1 cells also exhibited an increase after treatment of 40 mM D-serine, whereas we could not observe any significant change in C6 cells treated with D-serine. These results indicated that intracellular DAO is involved in the production of HPA from D-serine, leading to the astroglial cell death.

Detection of apoptosis in astroglial cells treated with HPA

In previous report, we reported that the astroglial cell death induced by D-serine treatment comprises

apoptosis (*2*). Therefore, Annexin V assay was performed 6, 12, 18 and 24 h after treatment with or without 1 mM Na-HPA and H_2O_2 in C6 cells to verify whether or not the decrease of the cell viability after HPA and H_2O_2 treatment was due to apoptosis. As shown in Fig. 7A, the treatment with Na-HPA caused an enhancement in levels of early apoptotic cells, stained only with Annexin V-FITC, and also late apoptotic cells, stained with both Annexin V-FITC and PI, in a time dependent manner. Incubation with Na-HPA for 18 h increased the ratio of early apoptotic cells to 69.1%, although that of control cells was 5.8%. The level of early apoptotic cells was increased in a time-dependent manner until 18 h after the treatment with Na-HPA, and then decreased at 24 h after the treatment. But the level of late apoptotic cells was increased in a time-dependent manner until 24 h after the treatment, and the ratio of late apoptotic cells reached to 10 times, compared with control.

The result of Annexin V assay of C6 treated with H_2O_2 (Fig. 7A) showed a slightly different pattern compared to that with HPA. The treatment with H_2O_2 also caused an enhancement in levels of early apoptotic cells and late apoptotic cells. The level of early apoptotic cells was increased to 43.4% at 6 h after the treatment with H_2O_2, and then decreased in a time-dependent manner until 24 h after the treatment. On the contrary, the level of late apoptotic cells was increased in a time-dependent manner until 24 h after the treatment, and the level of late apoptotic

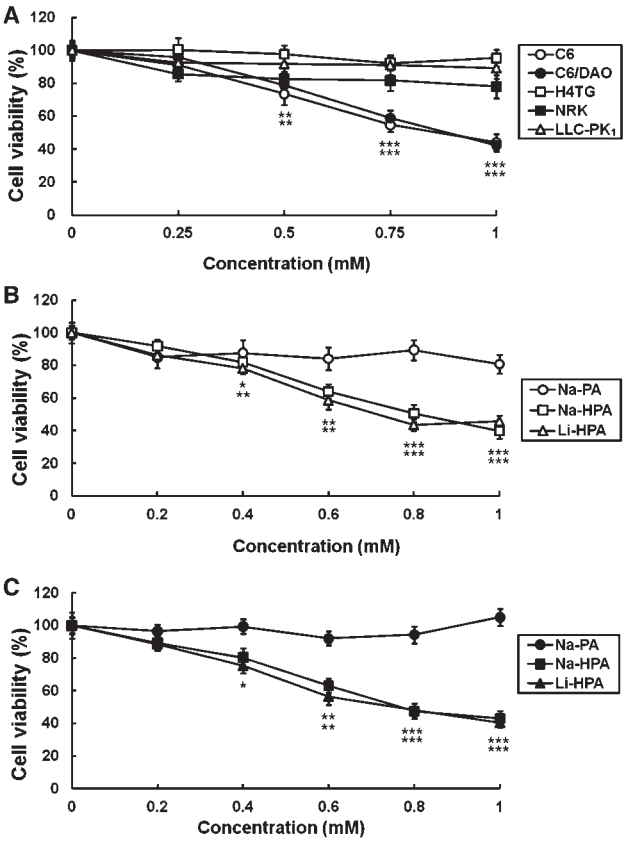

Fig. 5 Effects of HPA on cell viability. (A) The cell viability was evaluated by the means of MTS assay after treatment with the indicated concentrations of Na-HPA in C6 (open circles), C6/DAO (closed circles), H4TG (open squares), NRK-49F (closed squares) and LLC-PK_1 (open triangles) cells for 22 h. Results represent the mean ± SE of values obtained from six independent experiments. (B) C6 cells were incubated with indicated concentrations of two types of β-hydroxypyruvate, Na-HPA (open squares) and Li-HPA (open triangles) for 22 h. Na-pyruvate (open circles) was used as control. The cell viability was evaluated by the means of MTS assay. Results represent the mean ± SE of values obtained from six independent experiments. (C) C6/DAO cells were incubated with indicated concentrations of two types of β-hydroxypyruvate, Na-HPA (closed squares) and Li-HPA (closed triangles) for 22 h. Na-pyruvate (closed circles) was used as control. Cell viability was measured by means of the MTS assay. Results represent the mean ± SE of values obtained from six independent experiments. The viability of untreated cells was normalized to 100%, and the data are means ± SE of the percentage of controls. $*P<0.05$, $**P<0.01$ and $***P<0.001$, compared with control.

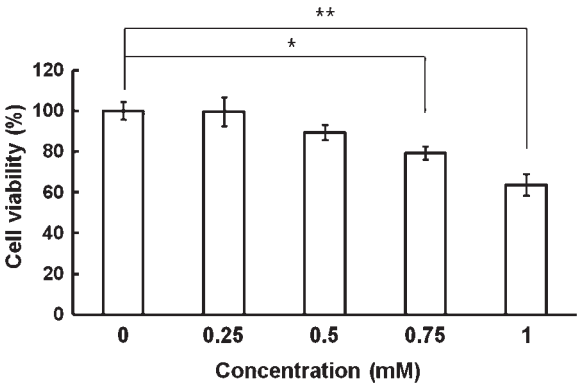

Fig. 6 Effects of HPA on cell viability of primary astrocytes. The cell viability was evaluated by the means of MTS assay after treatment with the indicated concentrations of Na-HPA in primary astrocytes for 22 h. Results represent the mean ± SE of values obtained from three independent experiments. The viability of untreated cells was normalized to 100%, and the data are means ± SE of the percentage of controls. $*P<0.05$ and $**P<0.01$, compared with control.

Table II. The detection of released NADH oxidizing compound 6 h after treating with various concentration of D-serine.

	0 mM D-serine	20 mM D-serine	40 mM D-serine
C6	197.93 ± 9.13 μM	199.94 ± 10.12 μM	201.43 ± 9.81 μM
C6/DAO	198.54 ± 6.24 μM	233.22 ± 10.50* μM	242.64 ± 12.75*,† μM
LLC-PK1	397.80 ± 7.52 μM	406.57 ± 6.01 μM	424.14 ± 2.64* μM

The data represents the mean ± SE of values obtained from four independent experiments.
$*P<0.05$, compared with 0 mM D-serine.
$^{\dagger}P<0.05$, comparison between C6 and C6/DAO.

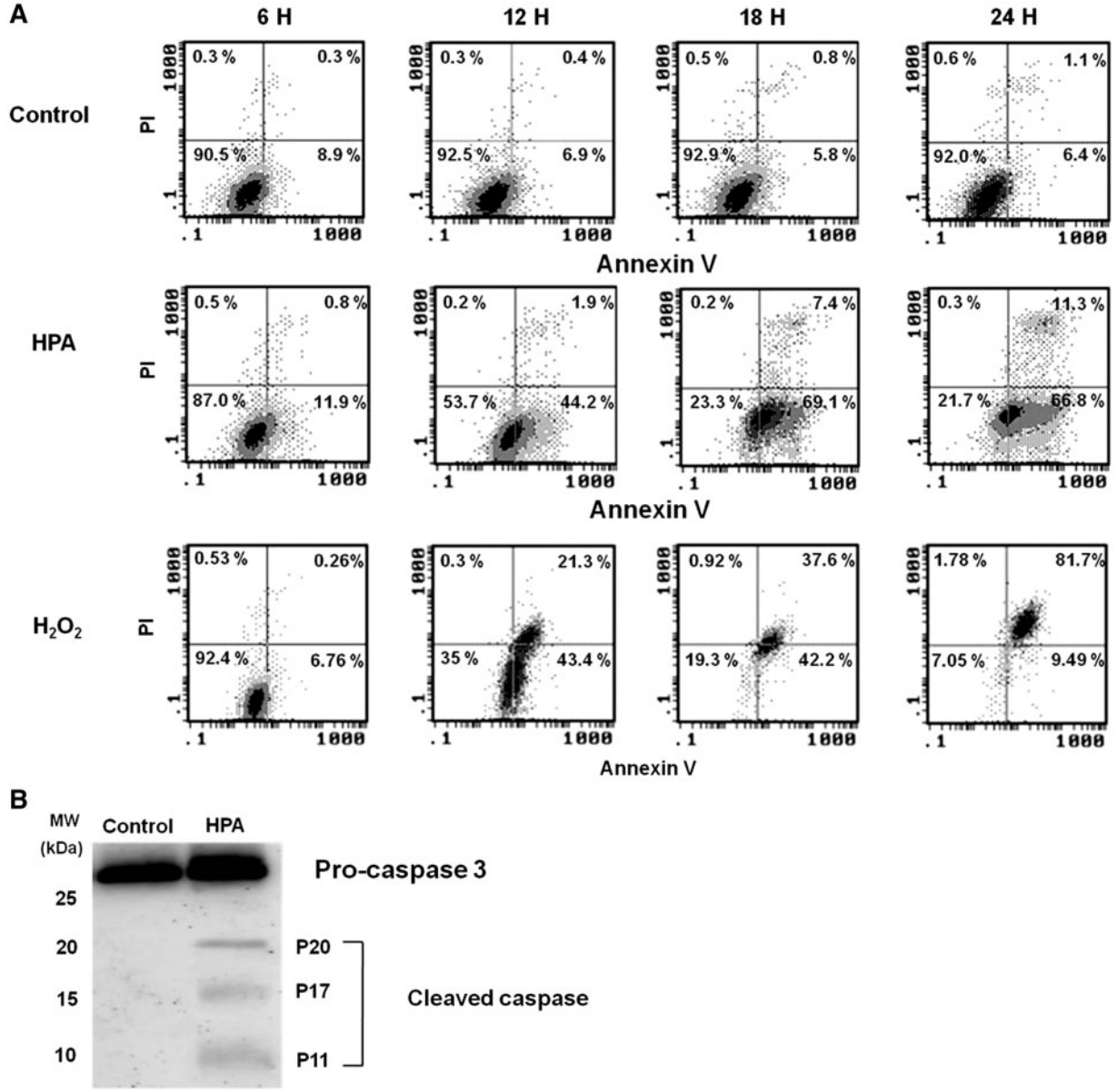

Fig. 7 Apoptosis in astroglial cells. (A) Annexin V assay was performed after treatment with or without 1 mM HPA and H_2O_2 for 6 h, 12 h, 18 h and 24 h. The harvested C6 cells were washed with binding buffer and then incubated with Annexin V-FITC and PI before being analyzed by flow cytometry. (B) Western blot analysis of caspase-3 expressed in C6 cells after treatment with or without 1 mM HPA for 24 h. Protein samples were separated by electrophoresis on 15% polyacrylamide gels. Anti-caspase-3 polyclonal antibody was used for the detection of the activation of caspase-3.

cells at 24 h after the treatment was reached up to 81.7%.

To confirm the HPA-induced apoptosis, we investigated the effect of Na-HPA on caspase-3 activation in C6 cells by Western blot analysis, since the activation of caspase-3 is commonly considered to cause apoptotic cell death. As shown in Fig. 7B, treatment of C6 cells with 1 mM Na-HPA induced activation of caspase-3, producing proteolytically cleaved caspase-3 subunits (P20, P17 and P11).

Taken together, these results suggest that the astroglial cell death induced by HPA treatment comprises apoptosis, as was observed by D-serine treatment previously (*2*).

Expression of GRHPR in several cell lines

It has been reported that HPA can be converted into D-glycerate by reduction with the enzyme called glyoxylate reductase/hydroxypyruvate reductase (GRHPR) (*29*), and this enzyme has been found in the liver and kidney at much higher levels than in other organs/tissues (*33*). Therefore, we investigated the mRNA expression of GRHPR in C6, C6/DAO, H4TG, NRK-49F and LLC-PK_1 cells. As shown in Fig. 8, the level of GRHPR mRNA was higher in H4TG (25-fold), NRK-49F (7-fold) and LLC-PK_1 (18-fold) than that in C6, whereas the level of GRHPR mRNA in C6/DAO was similar to that in C6 cells. These results indicated that not only DAO but also

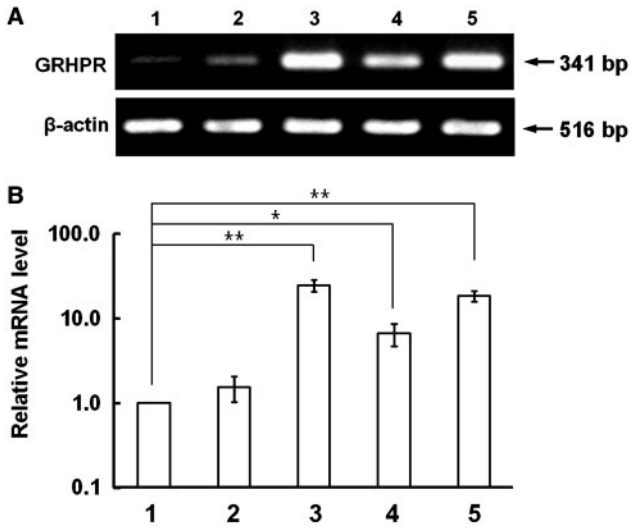

Fig. 8 Gene expression of GRHPR in C6, C6/DAO, H4TG, NRK-49 and LLC-PK$_1$ cells. Gene expression of GRHPR was measured in C6 (lane 1), C6/DAO (lane 2), H4TG (lane 3), NRK-49F (lane 4) and LLC-PK$_1$ cells (lane 5). (A) Total RNA was extracted from cultured C6, C6/DAO, H4TG, NRK-49F and LLC-PK$_1$ cells. Extracted total RNA was submitted to reverse transcriptase-PCR analysis, followed by electrophoresis on 2% agarose gel. (B) The level of GRHPR mRNA was measured by quantitative real-time PCR assay. Relative mRNA level of GRHPR was normalized by β-actin mRNA, and data are shown as the relative GRHPR mRNA level compared to C6 cells. Results represent the means ± SE of values obtained from three independent experiments. $*P<0.05$ and $**P<0.01$, compared with C6 cells.

catabolic enzyme of HPA such as GRHPR may be involved in the cytotoxicity induced by a high dose of D-serine treatment.

Discussion

The present study demonstrated the potential role of DAO in the metabolism of extracellular D-serine by the detection of cytotoxicity of its metabolites. In addition, our results provided the first description of the cytotoxic effect of HPA, the unique product of D-serine metabolism.

We showed previously that rat primary type-1 astrocytes exhibited increased cell death after D-serine treatment, and astroglial DAO activity was involved in this cytotoxic effect (*2*). This D-serine-induced toxicity is also observed in rat kidney, as reported that high dose of intraperitoneally injected D-serine damaged rat proximal straight tubules (*27*, *28*). DAO is also localized in the straight part of proximal tubules (*34*, *35*). Moreover, the administration of sodium benzoate, an inhibitor of DAO, attenuated D-serine-induced cytotoxicity and nephrotoxicity, suggesting that DAO activity was involved in the cytotoxic effect of a high dose of D-serine (*2*, *36*).

In the present study, we examined the effects of treatments with a high dose of D-serine and its metabolites on DAO-expressing cells derived from brain, liver and kidney in order to confirm the potential role of DAO in D-serine metabolism. As shown in Fig. 3, the cell viability of DAO-expressing cells treated with a high dose of D-serine was significantly decreased in a dose-dependent manner, although that of H4TG and NRK-49F cells was not significant. Moreover, cytotoxic effect of D-serine on DAO-overexpressing C6 cells (C6/DAO) was greater than that on C6 cells, indicating that intracellular DAO is involved in the cytotoxicity of D-serine. Furthermore, as shown in Fig. 3B, 20 mM sodium benzoate, a competitive inhibitor of DAO, significantly protected C6/DAO and LLC-PK$_1$ cells from D-serine, indicating that DAO activity is involved in the cell death induced by D-serine.

However, cytotoxic effect of D-serine on LLC-PK$_1$ cells was less than that on C6/DAO cells in spite of the higher expression level of DAO in LLC-PK$_1$ (Fig. 2). There may be some differences in the activity and/or substrate specificity between mouse (over-expressed in C6/DAO) and porcine DAO (expressed in LLC-PK$_1$), and the difference in the sensitivity to D-serine treatment may be due to the difference in the activity between mouse and porcine DAO. However, it has been reported that the maximum activity of the mouse recombinant DAO was estimated to be comparable with that of the porcine DAO synthesized in *E.coli* cells (*6*), and the affinity of mouse DAO enzyme for substrate is significantly lower than that of the porcine DAO enzyme (*37*). Based on these studies, we speculated that D-serine-induced cytotoxicity is dependent not only on intracellular level of DAO but also on cell characteristics such as sensitivity to cytotoxicity mediated by the metabolites.

In order to investigate the cytotoxic effects of the metabolites, which can be the candidates for the causes of D-serine-induced cytotoxicity, we examined the cell viability of DAO-expressing cells after the treatment of H_2O_2 and HPA. Hydrogen peroxide is one of the metabolites of D-serine and is regarded as major ROS, responsible for oxygen stress. It is a potentially harmful by-product of normal cellular metabolism that directly affects cellular functions and survival (*38*). To confirm that H_2O_2 was involved in D-serine-induced cell death, catalase and edaravone were examined based on their detoxifying activities against oxidative stress. Edaravone is a free-radical scavenger that has been evaluated as a neuroprotective compound, which reduces the increase of hydroxyl radical and superoxide anion level in several models of cerebral ischemia (*39*, *40*). It has been clinically used in Japan since 2001 to treat patients with cerebral ischemia. As shown in Fig. 4B and C, not only catalase but also edaravone significantly inhibited D-serine-induced cytotoxicity. Based on these results, we speculated that H_2O_2 is involved in D-serine-induced cell death due to the D-serine metabolism. It was also indicated that not only H_2O_2 but also other factor contributed to the D-serine-induced cell death of astroglial cells, because C6/DAO cells were partially recovered by catalase and edaravone.

Therefore, we investigated the cytotoxic effect of HPA, another product of D-serine metabolism. At first, we examined the production of HPA during the metabolism of D-serine. As shown in Table II, we could observe the elevated value of NADH oxidizing activity which is regarded as HPA after D-serine treatment on

C6/DAO and LLC-PK$_1$ cells. On the contrary, C6 cells treated D-serine did not exhibit significant change. Based on these results, we speculated that intracellular DAO was involved in the production of not only H_2O_2 but also HPA. However, it is difficult to determine how much of HPA was elevated in the cells from these experiments, because the regulatory system (s) governing the release and uptake of HPA has not been elucidated. It has been reported that ovine embryos incubated in medium containing 1.0 mM or 0.67 mM pyruvate, which has similar structure with HPA, showed average uptake values of 20.36 and 5.00 pmoles per embryo per 4 h, respectively (*41*). Therefore, we speculated that the transmembrane transport of HPA could be operated at lower level. Furthermore, we assumed that estimated value of HPA at 6 h after D-serine treatment was lower than the total amount of HPA produced by D-serine metabolism for 6 h, since HPA is known to be rapidly removed in all situations (*29*).

Next, as shown in Fig. 5, we could observe that HPA also exhibited a cytotoxic effect on astroglial cells in sharp contrast to pyruvate, suggesting that HPA also contributed to the cytotoxicity induced by a high dose of D-serine. We extended our studies from C6 (astroglial cell line) cells to primary astrocytes to confirm the uniqueness of effect of HPA on astroglial cells. As the results, we could observe that HPA treatment induced the death of primary astrocytes (Fig. 6). Moreover, we have observed that this astroglial cell death comprises apoptosis (Fig. 7). However, the cell viability of H4TG, NRK-49F and LLC-PK$_1$ cells was not affected by the addition of Na-HPA, suggesting that astroglial cells are more sensitive to HPA compared with other DAO-expressing cells derived from kidney and liver (Fig. 5A).

It is considered that metabolic pathway of HPA in astroglial cells is different from other cells. HPA has been reported to be converted into D-glycerate by reduction with GRHPR, and then D-glycerate participates in gluconeogenesis pathway via D-glycerate 3-phosphate (*29*). Moreover, it has been reported that GRHPR protein is predominantly expressed in liver and lesser amounts in kidney (*33*). This finding is consistent with our results (Fig. 8). We observed that the level of GRHPR mRNA was higher in H4TG, NRK-49F and LLC-PK$_1$ than those in C6 and C6/DAO, indicating that not only DAO but also catabolic enzyme of HPA may be involved in the cytotoxicity induced by a high dose of D-serine treatment. Alternative explanation for the difference in the sensitivity to HPA among the cell lines used in this study is that the amounts of HPA taken up into cells were different between the cell lines. However, the mechanism of HPA incorporation is still not completely clear. Therefore, detailed studies on the mechanism of HPA incorporation and differences in the mechanism between the cells are necessary to clarify the difference in the sensitivity to HPA among the cell lines. Based on these studies, we speculated that scarcity of catabolic enzyme of HPA such as GRHPR might lead to accumulation of HPA in the cell, which could be causative of cytotoxicity. It is tempting to speculate further that the cytotoxicity of HPA on C6 may be involved in the higher cytotoxic effect of D-serine on C6 than that on other DAO-expressing cells. Furthermore, based on the new finding that cytotoxicity of HPA was prominent for astroglial cells, we expect that HPA may be useful as therapeutic agent for the treatment of astroglial tumors.

Since D-serine is present at a micromolar concentration in the normal mammalian brain, the physiological relevance of D-serine-induced cytotoxic effect may seem unclear. The advantage of using a high dose of D-serine is that the involvement of DAO activity in the metabolism of D-serine can be detected through the cytotoxicity of the metabolite, H_2O_2 and HPA. It is possible that additional cellular stress, up-regulated biosynthesis or accumulation of D-serine in pathological conditions may influence the cytotoxic effect of D-serine, presumably through the production of H_2O_2 and HPA. Indeed, it has been reported that significant elevation (2~3 folds) in D-serine occurred during ischemia-reperfusion, even though elevated concentration of D-serine was as high as a micromolar concentrations (*42*). Furthermore, increases in D-serine levels were also observed in spinal cords of amyotrophic lateral sclerosis (ALS) patients (*43*). However, this study has not shown a quantitative concentration of D-serine. Therefore, detailed studies on the effects of D-serine in pathological conditions are necessary to clarify the D-serine-induced cytotoxic effect.

In conclusion, we have shown that DAO plays a key role in metabolism of extracellular D-serine. In addition, HPA, the particular product of D-serine metabolism by DAO, induced cytotoxic effect uniquely in astroglial cells. Further studies on D-serine – DAO system and its metabolites will help overcome diseases related to hyper- and hypofunction of NMDA receptor-mediated neurotransmission, such as stroke and schizophrenia.

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Conflict of interest

None declared.

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