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# D-Serine administration provokes lipid oxidation and decreases the antioxidant defenses in rat striatum

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ABSTRACT

The present work investigated the effects of intrastriatal administration of D-serine on relevant parameters of oxidative stress in striatum of young rats. D-Serine significantly induced lipid peroxidation, reflected by the significant increase of thiobarbituric acid-reactive substances, and significantly diminished the striatum antioxidant defenses, as verified by a decrease of the levels of reduced glutathione and total antioxidant status. Finally, D-serine inhibited superoxide dismutase activity, without altering the activities of glutathione peroxidase and catalase. In contrast, this D-amino acid did not alter sulfhydryl oxidation, a measure of protein oxidative damage. The present data indicate that D-serine *in vivo* administration induces lipid oxidative damage and decreases the antioxidant defenses in the striatum of young rats. Therefore, it is presumed that this oxidative stress may be a pathomechanism involved at least in part in the neurological damage found in patients affected by disorders in which D-serine metabolism is compromised, leading to altered concentrations of this D-amino acid.



D-Serine (D-Ser) is essentially formed from L-serine by serine racemase, which is present in rat and human tissues (Wolosker et al., 1999; Foltyn et al., 2005) and degraded by D-amino acid oxidase (Neims et al., 1966). Serine racemase can also catalyze the conversion of D-Ser into L-serine (Wolosker et al., 1999).

D-Ser is predominantly concentrated in the brain, particularly in the cerebral cortex, hippocampus, striatum and limbic forebrain. D-Ser distribution is positively correlated with serine racemase and with the glutamate and glycine sites at the N-methyl-D-aspartate (NMDA) receptor (Watanabe et al., 1992; Chouinard et al., 1993; Hashimoto et al., 1993; Nagata et al., 1994; Hamase et al., 1997). In addition, rat brain cells have a sodium-dependent and independent, saturable and temperature-sensitive uptake for D-Ser (Hayashi et al., 1997; Yamamoto et al., 2001; Ribeiro et al., 2002). It is also interesting to observe that the sodium-independent neutral amino acid transporter Asc1 shows a high affinity for D-Ser (Fukasawa et al., 2000).

A great body of evidence indicates that brain D-Ser is an important co-agonist for the NMDA glutamate receptor at the glycine-binding site and necessary for the physiological glutamate transmission (Danysz and Parsons, 1998). Alterations of D-Ser metabolism and function may potentially cause neurological and psychiatric symptoms, and seems to be involved in the pathophysiology of schizophrenia (Javitt, 2004), cerebral ataxia (Ogawa et al., 2003), learning ability in a dementia animal model (Danysz and Parsons, 1998), convulsion threshold (Danysz and Parsons, 1998), ischemic neuron death (Danysz and Parsons, 1998), amyotrophic lateral sclerosis, Alzheimer disease and other brain abnormalities (Wu et al., 2004; Hashimoto et al., 2005; Shoji et al., 2006; Bendikov et al., 2007; Sasabe et al., 2007). In addition, altered D-Ser levels are found in the brain of patients affected by nonketotic hyperglycinemia, a genetic disorder clinically characterized by convulsions, mental retardation and drowsiness (Iwama et al., 1997).

We have recently demonstrated that D-Ser elicits lipid peroxidation and protein oxidative damage and decreases glutathione (GSH) levels in rat brain *in vitro* (Da Silva et al., 2009). In the present study we investigated the effects of intrastriatal administration of D-Ser on relevant oxidative stress parameters, including thiobarbituric acid-reactive substances (TBA-RS), sulfhydryl content,

*Abbreviations:* CAT, catalase; D-Ser, D-serine; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); GPx, glutathione peroxidase; GSH, reduced glutathione; NMDA, N-methyl-D-aspartate; SOD, superoxide dismutase; SPSS, Statistical Package for the Social Sciences; TAS, total antioxidant status; TBA-RS, thiobarbituric acid-reactive substances.

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GSH levels, total antioxidant status (TAS) and the activities of the antioxidant enzymes glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD).

## 1. Experimental procedures

### 1.1. Animals and reagents

Wistar male rats of 30 days obtained from the Central Animal House of the Departmento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were used. The animals were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1$ °C) colony room, with food and water *ad libitum*. All reagents used were of analytical grade and purchased from Sigma Co. (St Louis, MO, USA).

### 1.2. D-Serine administration

The rats were deeply anesthetized with equitesine (3.33 mL/kg i.p.), which is a mixture of 0.25 M chloral hydrate, 88 mM magnesium sulfate heptahydrate, 10 mg/mL sodium thiopental, 5.8 M propylene glycol and 1.97 M ethanol, and placed in a stereotaxic apparatus. Two small holes were drilled in the skull for microinjection, and 1 μL of a 4.0 M D-Ser solution or 1 μL of 4.0 M NaCl (each solution prepared in water and pH was adjusted to 7.4 with NaOH), was slowly injected over 4 min into each striatum via needles connected by a polyethylene tube to a 10 μL Hamilton syringe. The needle was left in place for another 1 min before being softly removed, so that the total procedure lasted 5 min. The coordinates for injection were as follows: 0.6 mm posterior to the bregma, 2.6 mm lateral to the midline and 4.5 mm ventral from dura (Paxinos and Watson, 1986). The correct position of the needle was tested by previous injection of 0.5 μL of methylene blue solution (4% in saline) and further histological analysis. The experimental protocol was approved by the Ethics Committee for animal research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

### 1.3. Tissue preparation

Animals were killed by decapitation 30 min after intrastriatal injection of either D-Ser or NaCl. The brain was rapidly excised on a Petri dish placed on ice. The olfactory bulb, pons, medulla, cerebral cortex and cerebellum were discarded, and the striatum was dissected, weighed and kept chilled until homogenization with a ground glass type Potter-Elvehjem homogenizer in 10 volumes (1:10, w/v) of cold 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure oxidative stress parameters.

### 1.4. Protein determination

Protein content was determined in striatum supernatants by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

### 1.5. Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS levels according to the method described by Yagi (1998) with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid and 300 μL of 0.67% TBA in 7.1% sodium sulfate were added to 100 μL of tissue supernatant and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein.

### 1.6. Reduced glutathione (GSH) levels

GSH levels were measured according to Browne and Armstrong (1998). Tissue supernatants were diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/mg protein.

### 1.7. Total antioxidant status (TAS)

TAS, which represents the quantity of the tissue antioxidants, was determined by using a kit from RANDOX Laboratories. Striatum supernatants were incubated with ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) plus a peroxidase (metmyoglobin) and $H_2O_2$ to generate the cation $ABTS^+$, which produces a relatively stable blue-green color and is measured at 37 °C at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration (Miller et al., 1993; Yu and Ong, 1999). The results were expressed in mmol/mg protein.

### 1.8. Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel (1981) using tert-butyl-hydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH, and the supernatant containing 0.2–0.4 mg protein/mL. One GPx unit (U) is defined as 1 μmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein.

### 1.9. Catalase (CAT) activity

CAT activity was assayed according to Aebi (1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM $H_2O_2$, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.05–0.1 mg protein/mL. One unit (U) of the enzyme is defined as 1 μmol of $H_2O_2$ consumed per minute. The specific activity was calculated as U/mg protein.

### 1.10. Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund (1985) and is based on the capacity of pyrogallol to autoxidize, a process highly dependent on $O_2^{\bullet -}$, which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol and supernatants containing 0.1–0.2 mg protein/mL. A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The results are reported as U/mg protein.

### 1.11. Sulfhydryl content

This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid; DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 30 μL of 10 mM DTNB and 980 μL of PBS were added to 50 μL of striatum supernatants. This was followed by a 30-min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Results are reported as nmol TNB/mg protein.

### 1.12. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data was analyzed using the Student's *t*-test for unpaired samples. Only significant *t*-values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

## 2. Results

### 2.1. D-Ser intrastriatal administration induces lipid peroxidation in striatum

Initially we studied the effect of intrastriatal injection of D-Ser on TBA-RS levels in striatum 30 min after drug infusion. Fig. 1 shows that D-Ser administration induced lipid peroxidation (TBA-RS increase) in striatum [$t_{(8)} = 2.72$; $P < 0.05$]. Similar results were obtained after 2 h of D-Ser administration (results not shown).

### 2.2. D-Ser intrastriatal administration decreases non-enzymatic antioxidant defenses in striatum

GSH concentrations and TAS measured in striatum 30 min after D-Ser administration are shown in Fig. 2. It can be observed that D-Ser significantly diminished the concentrations of GSH (Fig. 2A) [$t_{(8)} = 7.62$; $P < 0.001$] and TAS (Fig. 2B) [$t_{(8)} = 3.21$; $P < 0.05$]. Taken