Serine racemase expression in mouse cerebral cortex after permanent focal cerebral ischemia[1]

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ABSTRACT

AIM: To study the alterations of the expressions of serine racemase in C57BL/6 mouse brain after permanent focal cerebral ischemia. **METHODS:** The mRNA level and the protein level of serine racemase were assayed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blot, respectively. The amount of *D*-serine and *L*-serine were measured by HPLC. **RESULTS:** High levels of serine racemase were constitutively expressed in the normal cortex of mouse. At early stage after middle cerebral artery occlusion (MCAO), no significant change in expression of serine racemase was observed in temporoparietal cortex in ipsilateral hemisphere. However, delayed transient decreases of serine racemase in both mRNA and protein levels were detected from d 6 to d 10 after ischemia. Correspondingly, *D*-serine concentration also declined in the ipsilateral cortex during this period when compared with the *D*-serine level in the contralateral cortex. **CONCLUSION:** Delayed decreases in serine racemase expression and *D*-serine level occurred in the temporoparietal cortex at the late stage after focal cerebral ischemia.

INTRODUCTION

D-serine is a selective and potent agonist for glycine site of *N*-methyl-*D*-aspartate (NMDA) receptor[1-3] with a potency similar to glycine[4,5], another endogenous agonist for glycine site of the NMDA receptor. In the striatum and medial prefrontal cortex, extracellular concentrations of *D*-serine are comparable to or even higher than those of glycine[6]. Immunohistochemistry and biochemical assays have revealed that *D*-serine is associated with protoplasmic astrocytes, a subtype of glial cells that closely apposed NMDA synapses[7,8]. Activation of non-NMDA glutamate receptor, the kainite subtype, invokes the release of *D*-serine from these astrocytes[8]. Selective destruction of the endogenous *D*-serine by applying *D*-amino acid oxidase (DAO) markedly decreases NMDA neurotransmission[7]. Since accumulative evidence has shown that the focal ischemia-induced neuronal damage involves an overstimulation of glutamate receptors, in particular those of the Ca^{2+}-conducting subtype sensitive to NMDA[9,10] and that NMDA receptor antagonist can protect delayed neuronal death in periinfarct region and attenuate ischemic damage[11,12], *D*-serine, as an endogenous co-agonist of NMDA receptor, might play an important role in the NMDA-mediated excitotoxicity in focal cerebral ischemia. *D*-serine is synthesized from *L*-serine by serine racemase, a pyridoxal 5'-phosphate-depen-

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dent enzyme[13]. Thus, serine racemase might be a potential target to develop effective treatment aimed at protecting the brain from the progression of ischemic injury. To clarify this possibility, one should know whether serine racemase is changed in the process of neuron injury and repair. In the present study, the expression of serine racemase in both mRNA and protein level were investigated in a permanent focal cerebral ischemia model in mice.

MATERIALS AND METHODS

Drugs and reagents Agarose, ethidium bromide, Keyhole Limpet hemocyanin (KLH), complete Freund's adjuvant, incomplete Freund's adjuvant and mouse monoclonal antibodies to β-actin were purchased from Sigma Chemicals Co (USA). TRI-REAGENT-LS extraction kit was purchased from Molecular Research Center Inc (USA). RNasin, dNTP, oligo$(dT)_{18}$ primer, and *Taq* DNA polymerase were obtained from Sangon Biotechnology Co (Canada). M-MuLV reverse transcriptase was from Fermentas Inc (Lithuania). Horseradish peroxidase (HRPO)-conjugated goat anti-rabbit antibody was obtained from CALBIOCHEM (USA). ECL chemiluminescence system and BCA protein assay kit were purchased from Pierce (USA). DEAE Sephadex A-50 was obstained from Pharmacia Inc. (Sweden). Polyclonal antibody to glial fibrillary acidic protein (GFAP) was purchased from Biogenex (CA)

Animals C57BL/6 male mice (Grade II, Certificate No 003, weighing 25±2 g,) obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences were kept in a temperature (25±2 ºC) and humidity (50 %-70 %)-controlled room, with a daily light cycle. All mice had free access to food and water.

Permanent focal cerebral ischemic model Permanent focal cerebral ischemia was achieved by electrocoagulation of the left middle cerebral artery (MCA) as previously described[14]. Briefly, mice were anesthetized intraperitoneally with chloral hydrate (500 mg/kg ip). A skin incision was made between the orbit and ear. The superior pole of the parotid gland was reflected downward, as was the temporalis after partial resection of its cranial insertion. The distal course of the MCA was then visible through the translucent skull. A small burr-hole craniectomy was performed with a dental drill. The MCA was coagulated by bipolar diathermy. The muscle and soft tissue were replaced and the incision was sutured. Mice were placed in a warm environment until they recovered from anesthesia. This procedure results in a reproducible ischemic lesion restricted to the temporoparietal cortex (ipsilateral to surgery) of operated animals. For sham operations, the MCA was exposed in the same way but not occluded. At different time intervals (1 h, 3 h, 8 h, 1 d, 2 d, 4 d, 6 d, 7 d, 8 d, 10 d, and 15 d) after MCAO or sham operation (4 d), mice were decapitated and the whole brains were removed. Both ischemic (the cortex ipsilateral to surgery) and contralateral temporoparietal cortices from the same mouse were dissected. Sham-operated mice were used as control.

Preparation of antibodies against serine racemase A peptide corresponding to the mouse serine racemase amino acid sequence, 72-85 (N'-EEKPKAVVTHSSGN-C') was synthesized (Genemed Synthesis, Inc, South San Francisco, USA) for use as immunogen. Peptide was conjugated to Keyhole Limpet Hemocyanin KLH essentially as described[15], using 0.1 % glutaraldehyde. Rabbit was immunized using KLH conjugates 1 mg in Complete Freund's Adjuvant for the first injection and 0.8 mg in Incomplete Freund's Adjuvant for subsequent boosts on d 21 and then every two weeks thereafter for four times in subcutaneous injection. After sixth injection, the antiserum was finally collected. The immunoglobulin was purified by ammonium sulfate fractionation and ion-exchange chromatography[16]. DEAE Sephadex A-50 was used for separating IgG from the majority of serum proteins. The specificity of the antibody was tested by immuno-blotting.

Measurement of serine racemase mRNA Total RNA was prepared from cortical samples with TRI-REAGENT-LS extraction kit according to the manufacturer's guidelines. For cDNA synthesis, 20 μL reverse transcription mixture containing total RNA 1 μg, $MgCl_2$ 10 mmol/L, dNTP 2 mmol/L, oligo $(dT)_{18}$ primer 1 μg, RNasin 20 U, M-MuLV reverse transcriptase 20 U were incubated in the reaction buffer at 37 ºC for 90 min and then the reverse transcriptase was inactivated by heating the reaction mixture to 95 ºC for 10 min. PCR was performed to assess the expression of serine racemase mRNA using β-actin as an internal control. Oligonucleotide primers specific for mouse serine racemase (Forward 5'-GTATACTGTGACCCAAGTGACG-3'; Reverse 5'-TAGACTGGTAGCAGTCATCTGC-3'; 288 bp) and β-actin (Forward 5'-GGTGTGATGGTGGGAATGGGTC-3'; Reverse 5'-CTTCTCCAGGGAGGAAGAGGATG-3'; 594 bp) were synthesized. PCR conditions were as follows: 1 μL of cDNA mix-

ture was subjected to amplification in 50 μL of final volume with $MgCl_2$ 2.5 mmol/L, dNTPs 200 μmol/L, 3 U of *Taq* DNA polymerase, and 50 pmol of each primer in the reaction buffer. For all of the reactions, preliminary experiments were performed to determine the number of PCR cycles at which saturation occurred, and the experiments mentioned were carried out with a number of cycles that precedes saturation. PCR cycles were as follows: warm up period of 5 min at 95 ºC, cycles of PCR (95 ºC for 45 s, 61 ºC for 50 s, 72 ºC for 1 min), and a final elongation period of 10 min at 72 ºC. The cycles were performed 29 times for serine racemase and 22 times for β-actin, respectively. PCR products 10 μL were separated by 2 % agarose gel electrophoresis and visualized using ethidium bromide staining. The density of each band was measured by a densitometer. This semiquantitative measure was expressed as ratios compared with β-actin.

Measurement of serine racemase Temporoparietal cortex from ipsilateral and contralateral hemisphere were homogenized with RIPA (NaH_2PO_4 9.1 mmol/L, Na_2HPO_4 1.7 mmol/L, NaCl 150 mmol/L, pH 7.4, 1 % IgepalCA-630, 0.5 % sodium deoxycholate, 0.1 % SDS) supplemented with PMSF, pepstatin, leupeptin, and aprotinin, and incubated on ice for 40 min. The homogenate was centrifuged at 20 000×*g* for 30 min at 4 ºC. Protein concentration in the supernatant solution was determined by BCA Protein Assay Kit. Protein samples in Tris-buffer loading solution (Tris-HCl 20 mmol/L (pH 6.8), 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.002 % bromophenol blue) were electrophoresed in 12 % polyacrylamide gels. The prestained proteins (80.9 kDa, 63.8 kDa, 49.5 kDa, 37.4 kDa, 26 kDa, 19.6 kDa) were used as molecular weight (M_r) standards. Proteins were then electrically transferred onto polyvinylidene fluoride membranes (Poll Corporation, Annarbor, MI, USA) in a semidry blotting apparatus. The transfer of protein to membranes was assessed by Ponceau S staining. Membranes were blocked with 5 % non-fat dried milk in TBS-T (Tris-HCl 20 mmol/L (pH 7.6), NaCl 137 mmol/L, 0.1 % Tween-20) overnight at 4 ºC, then with rabbit anti-mouse serine racemase antibody or antibodies to β-actin (1:5000 in TBS-T containing 2 % BSA) for 1 h at room temperature (RT), and finally with horseradish peroxidase (HRPO)-conjugated goat anti-rabbit antibody (1:10 000 dilution), for 1 h at RT. Signal detection was carried out using the ECL chemiluminescence system. Extensive washings with TBS-T were carried out between each step. The specificity of the antibody of serine racemase were tested on the cortex samples using the primary antibody after incubation with excess corresponding peptide overnight at 4 ºC.

Measurement of *D*-serine and *L*-serine Amino acid enantiomers were separated by HPLC using a carbon 18 reverse-phase column (250 mm) (Inertsil, GL sciences Inc, Tokyo, Japan) with fluorimetric detection after derivatization with *N*-tert-butyloxycarbonyl-*L*-cysteine (Boc-*L*-cys, Sigma) and *o*-phthaldialdehyde (OPA, Sigma), as described[19]. In brief, the tissue sample was homogenized in 10 volume of 5 % trichloroacetic acid (TCA) after addition of *L*-homocysteic acid (Sigma) as internal standard. An aliquot of the homogenate was dissolved in NaOH 0.1 mol/L and protein concentration was determined by BCA protein assay kit. After centrifugation at 18 000×*g* for 30 min at 4 ºC, to remove TCA, the supernatant was washed three times with water-saturated diethyl ether. The resultant sample was derivatized with Boc-*L*-Cys and OPA for 2 min at RT. The Boc-*L*-Cys/OPA derivatives were immediately applied to the HPLC system (Bioanalytical Systems, West Lafayette, IN, USA). Mobile phase A was MeCN-sodium acetate buffer 0.1 mol/L (pH 6.0) (9:91, v/v), and mobile phase B was MeCN-sodium acetate buffer 0.1 mol/L (pH 6.0) (16:84, v/v). A linear gradient was applied for 35 min from mobile phase A to B; then the elution was carried out with mobile phase B alone. The flow-rate was 1.4 mL/min. Fluorescence detection of the derivative of each amino acid was carried out at 443 nm with excitation at 344 nm[17].

Statistics Data were expressed as mean±SD and compared with Student *t*-test. Each group was performed in triplicate.

RESULTS

Alterations in serine racemase mRNA expression after cerebral ischemia Semiquantitative RT-PCR with β-actin as an internal standard was used to characterize the temporal profiles of serine racemase mRNA expression in the infarct cortex (Fig 1). High levels of serine racemase mRNA were constitutively expressed in the normal brain cortex. During the first four days after focal cerebral ischemia, serine racemase mRNA remained unchanged in the ipsilateral temporoparietal cortex. However MCAO caused a time-related gradual decrease of serine racemase expression in the ischemic cortex from d 6 to d 10 after occlusion. Serine

racemase mRNA level decreased markedly to 20 % and 10 % of the controls (P<0.01) at d 6 and d 7 respectively after ischemia (Fig 1B). Then, the serine racemase mRNA level returned gradually to the control level from d 10 to d 15 after ischemia.

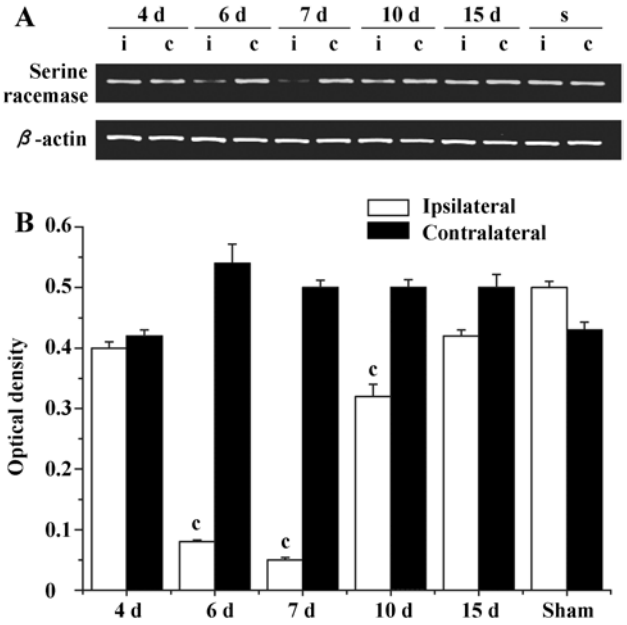

Fig 1. Serine racemase mRNA expression in C57BL/6 mice cortex after MCAO. A) Representative gel illustrating serine racemase mRNA expression in the ischemic cortex (i) and contralateral cortex (c) at different times after MCAO assessed by RT-PCR. The ubiquitous sequence β-actin was also studied and used as internal control. B) The ratio of serine racemase/β-actin was used to assess the mRNA levels of serine racemase in mouse cortex after MCAO. S, sham operation. n=5 for each time point. Mean±SD. ^cP<0.01 *vs* S.

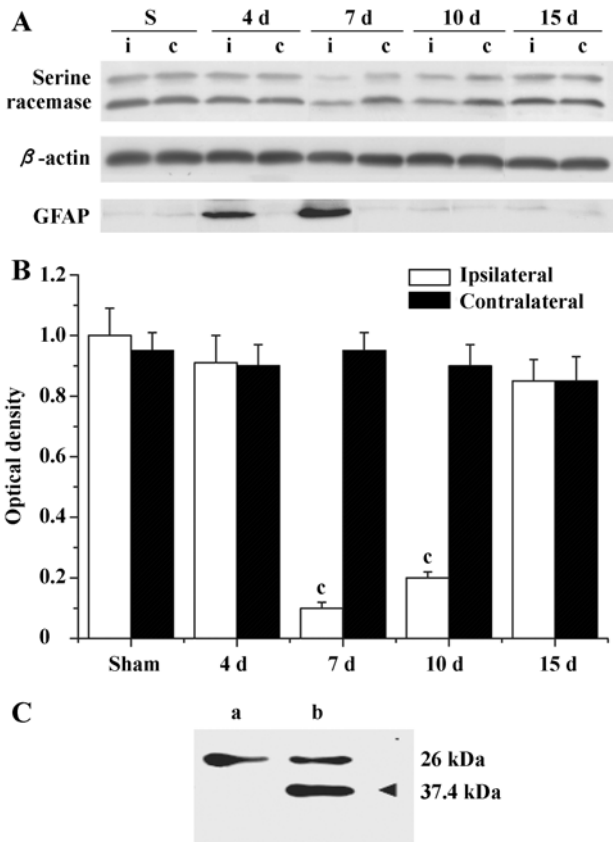

Fig 2. Serine racemase protein levels in C57BL/6 mice cortex after MCAO. (A) Representative time course of serine racemase expression in 70 μg of protein/lane of contralateral (c) and ipsilateral (i) temporoparietal cortex extracts from mice sham-operated (s) and mice at different time after MCAO were analyzed by western blot (the upper panel). Protein/lane of cortex extracts 30 μg were separated by sodium dodecyl sulfte-polyacrylamide gel electrophoresis and immunoblotted with monoclonal β-actin antibody (the middle panel). Protein/lane of cortex extracts 10 μg were immunoblotted with GFAP antibody (the lower panel). (B) The bar graph shows optical densities of serine racemase immunoblots in the contralateral and ipsilateral hemispheres after MCAO. n=5 for each time point. Mean±SD. ^cP<0.01 *vs* S. (C) Characterization of purified serine racemase antibodies by Western blot analysis. Antibodies against mouse serine racemase specifically recognized serine racemase as a band of about 37 kDa in cytosolic protein preparations of mouse cerebral cortex (70 μg/lane). Signal could be blocked by overnight preincubation with corresponding excess antigenic peptide (lane b). Lane b represents that the antibodies were overnight preincubation with PBS. The arrow indicates serine racemase.

Alterations of serine racemase protein levels after cerebral ischemia To further analyze whether a parallel change in the protein level of serine racemase occurred, the protein level of serine racemase in the ischemic region was quantified using Western blot analysis (Fig 2 A). Corresponding well with the results of RT-PCR analysis, significant decrease in the amounts of immunoreactive serine racemase protein in ischemic region were only observed between d 6 to 10 after ischemia. At d 15 after ischemia, the protein levels of serine racemase increased to the control level. In contrast, no significant alteration in the serine racemase protein level was observed in the ipsilateral temporoparietal cortex from 1 h to d 4 after ischemia. To evaluate the reactivity of astrocytes, the protein levels of glial fibrillary acidic protein (GFAP) in the cortex of mice were also examined after ischemia using Western blot analysis. During d 4 to d 8 after ischemia, significant increases of GFAP were observed in ipsilateral temporo-

parietal cortex (Fig 2A).

Antibody specific to the serine racemase By Western blot analysis, purified antibodies recognized two bands of about 37 kDa and 29 kDa in cytosolic protein preparations from mouse cortex. Preimmune serum could recognize the 29 kDa band in cytosolic preparations. But only 37 kDa band could be blocked completely by preadsorption with immunogenic peptide (Fig 2C). The results indicated that the antibody specifically recognized the serine racemase.

Alterations of *D*- and *L*-serine levels after cerebral ischemia To further assess whether the amount of *D*-serine was altered at the late stage after focal cerebral ischemia, *D*-serine content in temporoparietal cortex were measured from d 4 to d 15 after ischemia. To rule out the alteration of *D*-serine due to consequence of ischemia-induced cell death, *L*-serine concentrations in temporoparietal cortex were also measured. The ratio of *L*-serine relative to *D*-serine was rather constant in the cortex of control mice, and no difference was found in the ratio between two sides of cortex in control mice. However, at d 7 and d 10 after ischemia, the ratio of *L*-serine relative to *D*-serine in ischemic cortex was significantly increased to 3.4 and 2.5, respectively, compared with 1.9 in the sham-operated control (Tab 1). And the ratios of *L*-serine relative to *D*-serine in ipsilateral temporoparietal cortex at that time were markedly higher than that in contralateral cortex ($P<0.01$). In contrast, at d 4 and 15 after ischemia, no significant difference in the ratio of *L*-serine relative to *D*-serine or *D*-serine concentration were observed between ipsilateral and contrlateral hemisphere (Tab 1).

Tab 1. *D*-serine and *L*-serine content in C57BL/6 mice cortex after MCAO. *n*=5 to 6 for each time point. Mean±SD. $^{c}P<0.01$ *vs* S.

		D-serine/ μmol·g^{-1} protein	*L*-serine/ μmol·g^{-1} protein	*L*-/*D*-serine
Sham	i	3.23±0.30	6.39±0.44	1.96±0.03
	c	3.33±0.25	6.11±0.30	2.04±0.08
4 d	i	3.28±0.22	6.41±0.25	1.95±0.13
	c	3.44±0.27	6.46±0.28	1.85±0.11
7 d	i	1.73±0.29	6.15±0.26	3.48±0.09^{c}
	c	3.49±0.28	6.20±0.29	1.87±0.12
10 d	i	2.42±0.25	6.10±0.26	2.51±0.10^{c}
	c	3.11±0.24	6.16±0.29	1.98±0.09
15 d	i	3.10±0.23	6.10±0.27	1.85±0.11
	c	3.14±0.26	6.10±0.23	1.85±0.12

DISCUSSION

In this study, we evaluated the effects of permanent focal cerebral ischemia on temporoparietal cortex gene expression and protein levels for serine racemase, and examined the gross amount of *D*-serine. The following three principal findings emerged (1) The expression levels of serine racemase gene were altered in the ischemic cortex at the late stage after ischemia; (2) the levels of immunoreactive serine racemase protein in the ischemic cortex were decreased between d 6 and d 10 after ischemia; and (3) *D*-serine level also decreased in ipsilateral hemisphere at the late stage after ischemia compared with contralateral hemisphere or sham control. The changes in gene expression were paralleled closely by consistent changes in the immunoreactivity of serine racemase present in the ischemic cortex. Corresponding well with the decrease of serine racemase, marked increases of the ratio of *L*-serine relative to *D*-serine in ischemia cortex occurred at 7 d after MCAO. The reduction of *D*-serine in ischemic cortex seems to be a consequence of down-regulation of serine racemase at the transcription level during the late stage after ischemia. While an obvious neuronal injury was observed as early as 2 h post-MCAO and maximum infarction was found at d 1[18], the decline of serine racemase expression were only presented after d 4 post-MCAO when infarct size had decreased significantly from maximum infarction and reactive astrocyte had become more numerous[18]. It seems unlikely that cell death contributed to the reduction of serine racemase at the late stage of ischemia. Similarly, the reduction of *D*-serine in ischemic cortex at the late stage was unlikely to be a consequence of cell death since no alteration was found in *L*-serine content at the late stage. Although the antibody against serine racemase used in our study could recognize two bands of about 37 kDa and 29 kDa in cytosolic protein preparations from mouse cortex, the 29 kDa band was also detected in cytosolic preparations when preimmune serum was used. Thus, the 29 kDa band was a non-specific immune signal. Interesting, this non-specifically interacting protein was also decreased at d 7 in the ipsilateral cortex. It seems that the expression of this unknown protein might be effected by MCAO. Further studies are needed to characterize this protein.

D-serine and serine racemase are specificly enriched in protoplasmic astrocytes[3,8,19]. In this experiment we also examined the protein level of GFAP in the

temporoparietal cortex after MCAO using Western blot. In agreement with previous reports, significant increases of protein levels of GFAP were observed in ipsilateral hemisphere between d 4 to d 8 post-MCAO (Fig 2C). Although the mechanism of serine racemase down-regulation in the ipsilateral cortex after ischemia is not clear, the activation of astrocytes after ischemia may play important roles. Several groups have reported that reduction of NMDA receptor subunit in hippocampus after forebrain ischemia was observed at 7 d[20]. In our research, the reduction of *D*-serine also occurred at 7 d after ischemia. However, whether these alterations play roles in the ischemia-induced damage or repair process is still unknown.

Considerable interest has been placed on the potential involvement of NMDA receptors in the neurodegenerative process after cerebral ischemia. It has been suggested that an overstimulation of NMDA receptor may initiate the ischemic cascades and contribute to the damage occurred in the early stages of cerebral ischemia[21]. Our results that high levels of serine racemase and *D*-serine were only found at the early stage after ischemia suggest that *D*-serine in cortex may play important roles in the excitoxicity at the early stage of ischemia injury. Therefore, as a potential therapeutic target, serine racemase inhibition at the early stage, but not the late stage, might protect the brain from the progression of ischemic injury.

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