Direct Correlation Between Ischemic Injury and Extracellular Glycine Concentration in Mice With Genetically Altered Activities of the Glycine Cleavage Multienzyme System

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Background and Purpose—Ischemia elicits the rapid release of various amino acid neurotransmitters. A glutamate surge activates N-methyl-D-aspartate (NMDA) glutamate receptors, triggering deleterious processes in neurons. Although glycine is a coagonist of the NMDA receptor, the effect of extracellular glycine concentration on ischemic injury remains controversial. To approach this issue, we examined ischemic injury in mice with genetically altered activities of the glycine cleavage multienzyme system (GCS), which plays a fundamental role in maintaining extracellular glycine concentration.

Methods—A mouse line with increased GCS activity (340% of C57BL/6 control mice) was generated by transgenic expression of glycine decarboxylase, a key GCS component (high-GCS mice). Another mouse line with reduced GCS activity (29% of controls) was established by transgenic expression of a dominant-negative mutant of glycine decarboxylase (low-GCS mice). We examined neuronal injury after transient occlusion of the middle cerebral artery in these mice by measuring extracellular amino acid concentrations in microdialysates.

Results—High-GCS and low-GCS mice had significantly lower and higher basal concentrations of extracellular glycine than did controls, respectively. In low-GCS mice, the extracellular glycine concentration reached 2-fold of control levels during ischemia, and infarct volume was significantly increased by 69% with respect to controls. In contrast, high-GCS mice had a significantly smaller infarct volume (by 21%). No significant difference was observed in extracellular glutamate concentrations throughout the experiments. An antagonist for the NMDA glycine site, SM-31900, attenuated infarct size, suggesting that glycine operated via the NMDA receptor.

Conclusions—There is a direct correlation between ischemic injury and extracellular glycine concentration maintained by the GCS. (Stroke. 2007;38:2157-2164.)

Key Words: animal models ■ glutamate ■ glycine ■ neuroprotection ■ NMDA glutamate receptor ■ reperfusion ■ transgenic mice

An abnormal increase in extracellular glycine concentration, together with a rapid elevation of glutamate, is consistently elicited by ischemia.1 The elevation of glutamate leads to uncontrolled activation of N-methyl-D-aspartate (NMDA) receptors and intracellular penetration of calcium, which is followed by production of free radicals, mitochondrial dysfunction, DNA injury, and deleterious processes that finally lead to the demise of surrounding neurons.2 Activation of NMDA receptors is therefore considered a key process in the development of ischemic injury. Glycine is an inhibitory neurotransmitter in the brain stem and spinal cord,3 and it also plays a critical role as a modulator of NMDA receptors.2-4 The role of glycine in stroke remains controversial. In acutely prepared hippocampal slices, excitotoxicity and subsequent neuronal cell death could be induced by addition of high concentrations of glycine.5 These toxic effects were, however, observed only when a millimolar concentration of glycine was applied, whereas the peak level of extracellular glycine during ischemia was in the micromolar range.6 In contrast, high extracellular glycine failed to potentiate NMDA-evoked depolarization in vivo.7 Glycine protected neurons from hypoxia-induced toxicity in cortical neuron cultures.8 Recently, several antagonists at the glycine site of the NMDA receptor have been developed, and their neuro-
Osaka University (Osaka, Japan).17 Mutant GLDC cDNA with the test whether the delF756 mutation had a dominant-negative effect. A serum glycine level 1.8-fold higher than normal, which is considered tolial enzyme complex with 4 individual components: glycine oxidation of basal glycine concentrations. The GCS is a mitochon-
dribal enzyme complex with 4 individual components: glycine decarboxylase (GLDC), aminomethyl transferase, amino-
methyl carrier protein, and lipomamide dehydrogenase.14 GLDC is a homodimeric enzyme of ~200 kDa. An inherited
deficiency of either GLDC or aminomethyl transferase causes an inborn error of metabolism, glycine encephalopathy (GE), also called nonketotic hyperglycinemia.15 GE is characterized by neonatal coma and convulsions associated with the accumu-
lation of large amounts of glycine in cerebrospinal fluid, providing further evidence that the GCS plays a fundamental
role in maintaining extracellular glycine concentrations in the central nervous system.

To clarify the role of extracellular glycine in brain ische-
mia, we examined neuronal injury after transient occlusion of the middle cerebral artery (MCA) in mice with altered GCS
activities by monitoring extracellular amino acid concentra-
tions. Mice with altered GCS activities were generated by transgenic expression of normal GLDC or a dominant-
negative mutant of GLDC, which was previously found in a family with GE16 and characterized in this study. These
approaches have enabled us for the first time to elucidate a direct correlation between extracellular glycine concentra-
tions and ischemic injury.

Materials and Methods
Expression Analysis of GLDC cDNA in COS7 Cells
We previously identified a 3-base deletion, c.2266 to 2268delTTTC, in the GLDC gene in a GE family, which resulted in the deletion of 1 phenylalanine residue at amino acid position 756, delF756.16 The mother was a heterozygous carrier of the 3-base deletion and had a serum glycine level 1.8-fold higher than normal, which is considered the upper limit of the normal range. This observation prompted us to test whether the delF756 mutation had a dominant-negative effect. A 3.7-kb DNA fragment encoding human GLDC cDNA was subcloned into a pCAG vector (P-wild) for expression analysis (Figure 1A). The pCAG vector was kindly provided by Prof Jun-ichi Miyazaki of Osaka University (Osaka, Japan).17 Mutant GLDC cDNA with the delF756 mutation was also subcloned into pCAG (P-delF756). The pCAG vector containing β-galactosidase cDNA was prepared as a negative control. Plasmid DNAs of the pCAG vector, P-wild, P-delF756, and β-galactosidase were purified with a plasmid purifi-
mication mini kit (Qiagen GmbH, Hilden, Germany). Preparation of COS7 cells, transfection with lipofectamine (Invitrogen Corp, Carls-
bad, Calif), cell harvest, and assay of GLDC enzymatic activity were performed as described.16

Generation of Transgenic Mouse Lines
P-wild and P-delF756 plasmids were digested with SalI, and a 5.4-kb DNA fragment was recovered (Figure 1A). After purification of the DNA fragments, they were injected into fertilized eggs of BDF1 mice for generation of transgenic mice. Genomic DNA was purified from mouse tails with use of a DNeasy tissue kit (Qiagen), and a 201-bp DNA fragment of the CAG promoter region was amplified by polymerase chain reaction with a pair of primers, RBGP-1 and -2, for identification of the transgene. Nucleotide sequences of the primers were as follows: for RBGP-1, 5'-GCCCCTGGAGCATCTG ACTTC1GG-3’ and for RBGP-2, 5’-GACCTCTTTAAGGACACCTTT TG-3’. We mated the founder mice with C57BL/6 strain mice to obtain F1 mice and screened for cerebral glycine concentration. Transgenic mice of 2 selected lines, high-GCS and low-GCS mice, were back-
crossed with C57BL/6 mice 10 times and used for the following studies.

Enzymatic Analysis of GLDC and GCS Activity
GLDC enzymatic activity was determined by an exchange reaction between CO2 and glycine with NaH[14C]O3 as described.16 GCS activity was measured by a decarboxylation reaction with [1-14C]glycine as described.13

Regional Cerebral Blood Flow Measurement
Regional cerebral blood flow was measured by the laser-Doppler flowmeter method with a Laserflo BPM2 (Vasamedics, St. Paul, Minn). The flowprobe (0.5-mm diameter) was placed on the cranial bone above the MCA territory (0.5 mm posterior and 4 mm lateral from the bregma) and away from large surface vessels. Steady-state baseline values were recorded before MCA occlusion, and blood flow data were then expressed as percentages of preocclusion baseline. No significant difference in the percent change in cerebral blood flow values was detected during and after ischemia (supple-
mental Figure 1, available online at http://stroke.ahajournals.org).

Induction of Focal Cerebral Ischemia
Males of high-GCS, low-GCS, and control C57BL/6 mice lines weighing 25 to 30 g were used for the ischemia study. Anesthesia was induced with 2% halothane in a closed chamber and maintained with 1.0% to 1.5% halothane in 30% O2 and 70% N2O delivered via facemask. Rectal temperature was monitored and maintained at 37±0.5°C with a thermal blanket throughout the surgical procedure. Focal cerebral ischemia was induced by MCA occlusion by the intraluminal suture technique.18 A 5–0 nylon monofilament suture with a round tip was inserted into the internal carotid artery 11±0.5 mm from the bifurcation of the common carotid artery until the laser-Doppler flowmeter signal abruptly dropped. After 60 minutes of MCA occlusion, the nylon suture was removed and blood flow restoration was confirmed by the laser-Doppler flowmeter signal. Mice in which the laser-Doppler flowmeter signal during ischemia exceeded 10% of the preischemic signal were excluded from this study. All experiments and surgical procedures were approved by the Akita University Animal Care and Use Committee.

In Vivo Microdialysis
Twenty-four hours before MCA occlusion, vertical microdialysis probes (0.22-mm outer diameter, 2-mm membrane length; Eicom Corp, Tokyo, Japan) were stereotaxically implanted in the left striatum of mice under anesthesia. The probe was coordinately
implanted at 0.6 mm anterior and 2.0 mm lateral to the bregma and 2 mm ventral from the brain surface, according to the 1997 atlas of Franklin and Paxinos. The external portions of the probes were fixed to the skull with dental cement. Throughout ischemia, dialysis probes were perfused with Ringer’s solution (147 mmol/L NaCl,
2.3 mmol/L CaCl₂, 4.0 mmol/L KCl; pH 7.0) at a rate of 2 μL/min. The microdialysate (20 μL) was collected every 10 minutes. After a stabilization period of 1 hour, the samples were collected from 1 hour before MCA occlusion to 2 hours after MCA occlusion. Amino acid concentrations in the samples were measured by high-performance liquid chromatography (Eicom Corp).

**Administration of SM-31900**
An inhibitor of the glycine binding site of the NMDA receptor, SM-31900 (Sumitomo Pharmaceuticals Co Ltd, Osaka, Japan), was dissolved in physiological saline, which was then adjusted to pH 8.5. The animals subjected to MCA occlusion were randomly assigned to vehicle or SM-31900 treatment groups. Vehicle or SM-31900 (10 mg/kg IV) was administered twice at 30 and 10 minutes before MCA occlusion.

**Measurement of Infarct Size and Infarct Volume**
Twenty-four hours after MCA occlusion, the mice were deeply anesthetized and decapitated. The brain was removed and sectioned coronally into four 2-mm slices with a mouse brain matrix (Harvard Apparatus, Cambridge, Mass). The slices then were placed in 2% 2,3,5-triphenyltetrazolium chloride solution at 37°C for 10 minutes and fixed in a 10% buffered formalin solution. The infarct area, stained white, was measured with NIH Image analysis software, and infarct volume was calculated by summing the infarct volumes of sequential 2-mm-thick sections. Infarct volume was measured in different groups of animals from those used for microdialysis studies because infarct volume cannot be evaluated after probe insertion for microdialysis.

**Statistics**
All data were expressed as mean±SD. The statistical differences in regional cerebral blood flow and amino acid concentrations among and within the mouse groups were analyzed by a 1-way ANOVA and Dunnett’s test for multiple comparisons. Significance was accepted with P<0.05.

**Results**

**Identification of Dominant-Negative GLDC**
When 1 μg of plasmid with normal human GLDC cDNA (P-wild) was expressed in COS7 cells, the specific GLDC
activity was 9.8±0.8 nmol of glycine formed per milligram protein per hour, which was defined as 100% GLDC activity (Figure 1B). Expression of 2 µg of P-wild plasmid resulted in 215.8±19% GLDC activity. Negligible GLDC activity was detected in transfection of 1 µg of P-delF756 plasmid.10 GLDC activity was reduced to 62.1±0.3%, 47.0±1.9%, and 32.0±1.6% in cotransfection with 1, 2, and 4 µg P-delF756 plasmid together with 1 µg P-wild plasmid, respectively (Figure 1B). No reduction in GLDC activity was observed when 1 µg β-galactosidase plasmid was cotransfected. Because GLDC activity was inhibited in response to amounts of P-delF756, we concluded that the delF756 mutation had a dominant-negative effect.

**Generation and Biochemical Characterization of the High-GCS Mouse Line**

Normal GLDC cDNA and mutant GLDC cDNA with the delF756 mutation were subjected to transgenic expression in mice under control of the CAG promoter (Figure 1A). A SalI/SalI fragment (5.4 kb) of P-wild plasmid containing normal human GLDC cDNA was injected into 50 fertilized eggs. A total of 13 mice were born. One of them turned out to carry the transgene. It grew normally and was fertile to establish a transgenic mouse line. Mice of this line had significantly lower cerebral glycine concentrations (0.71±0.06 µmol/g wet tissue) than did wild-type C57BL/6 mice (0.90±0.05) as shown in Figure 2A. The enzymatic activity of the GCS was determined by a glycine decarboxylation reaction in tissue samples of mouse cerebrum. GCS activity was 0.48±0.14 nmol of CO₂ formed per milligram protein per hour, which was 340% of that of wild-type C57BL/6 mice (0.14±0.03). This transgenic mouse line was designated high GCS.

**Generation and Biochemical Characterization of the Low-GCS Mouse Line**

A SalI/SalI fragment (5.4 kb) of the P-delF756 plasmid containing mutant human GLDC cDNA (Figure 1A) was injected into 225 fertilized eggs, and 72 mice were born. The transgene was identified in 15 of 72 mice. By 3 months of age, 5 of 15 founder mice had died of unknown causes. We observed that 1 of those 5 mice exhibited shivering and loss of body weight at 3 month of age. Measurement of amino acid contents in the brain sample from that mouse showed a marked increase in glycine content, 3.2 nmol/g tissue in the cortex. We assumed that founder mice with massive glycine accumulations died at an early age, whereas founder mice with no or moderate glycine accumulations survived. The remaining 10 founder mice grew normally and were fertile. These founder mice were mated with wild-type C57BL/6 mice, and their F₁ offspring were screened for brain glycine content. The mouse line with the highest glycine content had 1.36±0.06 µmol glycine per gram of tissue in the cerebral cortex (Figure 2A), in which glycine content in the striatum was also significantly high (Figure 2B). GCS activity in the mouse cerebrum was 0.04±0.01 nmol of CO₂ formed per milligram protein per hour, which was 29% of that of control C57BL/6 mice (0.14±0.03). This mouse line was designated low GCS. Histological examination revealed no abnormality in either high-GCS or low-GCS mice (data not shown).

**Basal Concentrations of Extracellular Amino Acid Neurotransmitters**

Glycine concentrations in microdialysates were 11.7±1.0, 7.3±0.9, and 16.6±1.0 pmol/20 µL in wild-type C57BL/6, high-GCS, and low-GCS mice, respectively (Figure 2C). Extracellular glycine concentrations were therefore estimated as 1.0±0.1, 1.4±0.1, and 0.6±0.1 µmol/L in wild-type, low-GCS, and high-GCS mice, respectively, based on the sampling efficiency of our microdialysis system. Glutamate concentrations in microdialysates of wild-type C57BL/6, high-GCS, and low-GCS mice were 5.0±0.8, 5.4±0.7, and 4.8±1.5 pmol/20 µL, respectively (Figure 2D), which corresponded to 0.4±0.1, 0.5±0.1, 0.4±0.1 µmol/L, respectively, in extracellular glutamate concentration. The glycine and glutamate concentrations in wild-type mice showed good agreement with those in a previous report.6 The extracellular concentration of glycine was significantly (P<0.01) higher or lower in low-GCS or high-GCS mice, respectively, compared with wild-type mice. No significant difference was observed among the 3 mouse lines in terms of extracellular concentrations of glutamate, taurine, alanine, or γ-aminobutyric acid (GABA), as shown in Figures 3C through 3E.
Profiles of Extracellular Amino Acid Concentrations in MCA Occlusion

The glycine concentrations in all groups increased significantly at 20 minutes after MCA occlusion and reached their peak at 10 minutes after reperfusion (Figure 3A). The peak concentrations were 63.4±10.0, 36.6±12.0, and 31.5±7.9 pmol/20 µL in low-GCS, C57BL/6, and high-GCS mice, respectively. During and after MCA occlusion, glycine concentrations were persistently higher in low-GCS mice and lower in high-GCS mice compared with control mice. The glutamate concentrations were elevated in all 3 groups at 20 minutes after MCA occlusion and eventually reached their peak (247.7±35.9, 241.2±31.0, and 165.4±92.0 pmol/20 µL in low-GCS, C57BL/6, and high-GCS mice, respectively) at 10 minutes after reperfusion (Figure 3B). No significant difference in glutamate concentration was observed among the 3 groups.

Figure 3. Extracellular concentrations of amino acid neurotransmitters in focal ischemia. A, The glycine concentration was significantly higher and lower in the low-GCS (n=10) and high-GCS (n=10) group, respectively, compared with controls (n=10). There were no significant differences in extracellular concentrations of glutamate (B), taurine (C), alanine (D), or GABA (E) among the 3 groups. The horizontal bar stands for the MCA occlusion period (60 minutes). Values are mean±SD. *P<0.05, **P<0.01.
throughout the experiments. MCA occlusion caused significant elevations of extracellular concentrations of taurine, alanine, and GABA (Figures 3C through 3E). There were no significant differences in extracellular taurine, alanine, or GABA concentrations among 3 mouse groups at each time point.

Infarct Size

In C57BL/6 control mice, infarct areas were mainly in the cortex and striatum (Figure 4A). The infarct areas extended to the whole MCA territory in low-GCS mice, whereas the areas were confined to the striatum in high-GCS mice. The infarct area in low-GCS mice was significantly larger than that of control mice in all slices, whereas the area of 4- and 6-mm slices was significantly smaller in high-GCS mice compared with controls (Figure 4B). As shown in Figure 4C, infarct volume in low-GCS mice after MCA occlusion was significantly increased by 69%, and that in high-GCS mice was significantly reduced by 21% compared with wild-type mice (low-GCS mice, 121.6 ± 19.0 mm³; control C57BL/6 mice, 71.6 ± 29.1 mm³; high-GCS mice, 56.5 ± 7.9 mm³).

Effect of the NMDA Receptor Glycine Site Antagonist SM-31900 on Infarct Size

Compared with vehicle-treated mice, infarct regions in the striatum and cortex were smaller in the SM-31900–treated
group in both low-GCS and control mice (Figure 5A). Pretreatment with SM-31900 significantly reduced infarct volume in C57BL/6 and low-GCS mice by 35% and 42%, respectively (control C57BL/6, 46.3 ±18.6 mm³; low-GCS mice, 69.8±24.4 mm³; Figure 5B). Physiological parameters during ischemic experiment are summarized in supplemental Table I, available online at http://stroke.ahajournals.org.

Discussion
We generated 2 transgenic mouse lines with genetically altered GCS activities, i.e., high-GCS and low-GCS mice, and examined neural injury after MCA occlusion by monitoring concentrations of extracellular amino acids. Low-GCS mice had higher extracellular glycine concentrations and larger infarct volumes than did control mice. In sharp contrast, high-GCS mice had lower extracellular glycine levels and smaller infarct volumes. In the development of ischemic injury, a high extracellular concentration of glutamate is known to be neurotoxic,2,21 whereas GABA plays a neuroprotective role.22 In our experiments, no significant differences in extracellular glutamate or GABA concentrations were observed among the 3 mouse groups with distinct extracellular glycine concentrations. These results demonstrated a direct correlation between neuronal injury and extracellular glycine concentration, which is maintained by the GCS.

Glycine plays 2 important roles in the central nervous system: that of an inhibitory neurotransmitter and that of a modulator of excitation at the NMDA receptor. The infarct volume in low-GCS mice was markedly reduced by administration of an antagonist of the NMDA receptor glycine site. One possible explanation for this result is that low GCS activity affected ischemic damage mainly via the NMDA receptor. Lower GCS activity caused a higher extracellular glycine concentration, which resulted in overexcitation of NMDA receptors, leading to more severe ischemic injury. If SM-31900 had completely blocked the glycine site of the NMDA receptor, then infarct volume in SM-31900–treated wild-type mice would have been similar to that of SM-31900–treated, low-GCS mice. However, infarct volume in SM-31900–treated, low-GCS mice was similar to that of untreated, wild-type mice (Figure 5), which may suggest partial blocking of the glycine site by SM-31900 or the presence of another neuroprotective effect of SM-31900 that remains unidentified. At this moment, we cannot exclude the possibility that the altered GCS activity affected neural injury via inhibitory glycine receptors. Further study is required to understand the mechanism underlying a direct correlation between ischemic injury and extracellular glycine concentrations.

It is an old but still open question whether the glycine site of the NMDA receptor is saturated under physiological conditions.23,24 Glycine is normally present in brain interstitial space at a concentration of 4 μmol/L in the cortex and 1 μmol/L in the striatum.6,25 The NMDA-associated glycine-binding site is fully saturated at a glycine concentration of <1 μmol/L, suggesting that the glycine site of the NMDA receptor should be saturated under physiological conditions.5 In line with these results, high extracellular glycine concentrations failed to potentiate NMDA-evoked depolarization in vivo by microdialysis with extracellular field potential record-

ing.7 Many in vivo studies have nevertheless demonstrated protective effects of NMDA glycine site antagonists.9,24 In this study, we showed that infarct volume was larger in mice with higher extracellular glycine concentrations and that neural injury was ameliorated by an NMDA glycine site antagonist. Recently, we found that low-GCS mice had behavioral abnormalities such as hyperactivity and increased aggression. These phenotypes resemble the symptoms of patients with a mild form GE, who do not manifest neonatal seizures or coma but instead have behavioral abnormalities.26 Thus far, these observations support the notion that the glycine-binding site of the NMDA receptor is not functionally saturated under physiological conditions and that the NMDA receptor could respond to changes in extracellular glycine concentrations.

Extracellular glycine reached its highest peak during MCA occlusion in low-GCS mice compared with controls (Figure 3A). Ischemia-induced acidosis leads to dysfunction of the glycine transporter, which triggers an efflux of intracellular glycine into the extracellular space.27 The higher peak was probably due to a higher intracellular glycine content in the striatum in low-GCS mice (Figure 2B). The peak glycine level in high-GCS mice was not lower than that in controls 50 to 60 minutes after MCA occlusion, which may be explained by the fact that the glycine content in the striatum did not significantly differ between high-GCS and wild-type mice (Figure 2B). The elevated glycine level was normalized more rapidly in high-GCS mice compared with low-GCS and wild-type mice (Figure 3). Total glycine release during the first 2 hours after ischemia may affect the extent of ischemic injury rather than the glycine concentration at each time point.

An antagonist of the glycine site of the NMDA receptor, SM-31900, ameliorated ischemic injury in high-GCS mice, in accordance with a previous report that SM-31900 attenuated neuronal injury in a rat model of focal ischemia.19 A number of antagonists for the glycine site of the NMDA receptor have been developed to date.9 Clinical trials with some of the antagonists have been performed, but so far, these have proven unsuccessful. The infarct size in high-GCS mice (56.5±7.94 mm³) was comparable to that of SM-31900–treated, wild-type C57BL/6 mice (46.3±18.6 mm³) as shown in Figures 4C and 5B, suggesting that the enhancement of GCS activity has a similar neuroprotective effect. If a small molecule that enhances GCS activity were to become available, it could be used as a neuroprotective drug for various NMDA receptor–related neurodegenerative disorders. The low-GCS and high-GCS mouse lines established in the current study would be valuable tools for studying the effect of extracellular glycine concentrations in vivo.

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Disclosures
None.

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