Cerebellar Glutamine Synthetase in Children After Hypoxia or Ischemia

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Background: Glutamate has been implicated in the pathophysiology of acute hypoxic-ischemic encephalopathy. Glutamine synthetase is an enzyme found in astrocytes that converts glutamate to its nontoxic analogue, glutamine. The present study tests the hypothesis that brain glutamine synthetase activity increases in response to acute hypoxic-ischemic insults and not in response to chronic hypoxia-ischemia or non–hypoxic-ischemic neurological disease.

Summary of Report: Frozen sections of cerebellum from children who died with acute or chronic hypoxic-ischemic insults or chronic non–hypoxic-ischemic neurological disease were spectrophotometrically assayed for glutamine synthetase activity by an observer who was blinded to the clinical group assignment of each specimen. Enzyme activity was elevated in specimens from children with acute hypoxic-ischemic insults (mean 6.5; range 5.4–7.2 units/g wet tissue wt) as compared with those from patients with chronic hypoxia-ischemia (mean 2.8; range 0.7–10.2 units/g wet tissue wt) or with non–hypoxic-ischemic neurological disease (mean 2.6; range 1.3–3.9 units/g wet tissue wt). This difference was not due to differences in the degree of histological astrocytosis or edema among the specimens. Statistical analysis by the Kruskal-Wallis one-way analysis of variance by ranks test indicates that the three data groups do not come from one population (p < 0.05).

Conclusions: These results support the notion that glutamine synthetase activity increases in response to acute hypoxic-ischemic nervous system injury in children and that other compensatory mechanisms prevail in the case of chronic hypoxic-ischemic insults. (Stroke 1991;22:1312–1316)

Glutamine synthetase is the primary enzyme responsible for detoxication of glutamate in the central nervous system. It converts this excitotoxin to its nontoxic metabolite, glutamine.1 It is largely localized to astrocytes and, in the brain, is present in high concentration in the cerebellum.2 Glutamate has been implicated in the pathophysiology of acute hypoxic-ischemic encephalopathy.3 Astrocytes are often found in abundance in areas of acute hypoxic-ischemic insult and are thought to play a role in “rescuing” the surrounding neurons from the metabolic by-products of such injury.4 Petito et al5 have noted an increase in brain glutamine synthetase in rats subjected to acute bilateral carotid occlusion. We have measured glutamine synthetase activity in the cerebellum of 20 pediatric patients with acute or chronic hypoxic-ischemic injury or with neurological disease in which hypoxic-ischemic injury did not play a role. The present study demonstrates our ability to differentiate between acute hypoxic-ischemic injury and the other conditions, based on these enzyme levels.

Materials and Methods

Patient samples were divided into three categories: acute, those coming from patients with no prior history of neurological, cyanotic or ischemic cardiac, or respiratory disease who sustained an acute cardiorespiratory arrest (n = 3); chronic, those coming from patients with a history of complex congenital heart disease or extracorporeal membrane oxygenation (duration 4–21 days), without a history of a defined clinically acute neurological or cardiorespiratory event (n = 13); and non–hypoxic-ischemic, those coming from patients with central neurological disease not associated with cardiorespiratory failure (n = 4). Table 1 provides the clinicopathologic profile of each patient from whom a specimen was obtained for analysis. Representative case histories are given in “Results.” Autopsies were performed in the Pathology Department of Children’s Hospital of Pittsburgh within 24 hours after death. In patients with acute cardiac or respiratory arrest, routine histopathologic staining demonstrated variable degrees of astrocyto-
TABLE 1. Clinical Profiles of Patients From Whom Cerebellar Samples Were Obtained for Analysis of Glutamine Synthetase Activity

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical diagnosis</th>
<th>Age at death</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cardiac arrhythmia/arrest</td>
<td>9 yr</td>
<td>M</td>
</tr>
<tr>
<td>2</td>
<td>Suspected sepsis (+ postmortem blood culture only)</td>
<td>3 days</td>
<td>F</td>
</tr>
<tr>
<td>3</td>
<td>Epiglottitis</td>
<td>2 yr</td>
<td>M</td>
</tr>
<tr>
<td>Chronic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Pulmonary fibrosis/ECMO</td>
<td>5 yr</td>
<td>F</td>
</tr>
<tr>
<td>2</td>
<td>Pulmonary atresia, right ventricular hypoplasia/ECMO</td>
<td>3 mo</td>
<td>M</td>
</tr>
<tr>
<td>3</td>
<td>DiGeorge syndrome/ECMO</td>
<td>1 mo</td>
<td>F</td>
</tr>
<tr>
<td>4</td>
<td>Transposition of great arteries, ventriculoseptal defect, coarctation of aorta</td>
<td>17 days</td>
<td>M</td>
</tr>
<tr>
<td>5</td>
<td>Diaphragmatic hernia/ECMO</td>
<td>5 days</td>
<td>F</td>
</tr>
<tr>
<td>6</td>
<td>Transposition of great arteries, patent ductus arteriosis</td>
<td>6 hr</td>
<td>M</td>
</tr>
<tr>
<td>7</td>
<td>DiGeorge syndrome, tetralogy of Fallot</td>
<td>1 mo</td>
<td>M</td>
</tr>
<tr>
<td>8</td>
<td>Diaphragmatic hernia/ECMO</td>
<td>11 days</td>
<td>M</td>
</tr>
<tr>
<td>9</td>
<td>Tetralogy of Fallot</td>
<td>3 yr</td>
<td>M</td>
</tr>
<tr>
<td>10</td>
<td>Atrioventricular canal</td>
<td>4 mo</td>
<td>F</td>
</tr>
<tr>
<td>11</td>
<td>Cardiomyopathy</td>
<td>1 mo</td>
<td>M</td>
</tr>
<tr>
<td>12</td>
<td>Venticuloseptal defect, hypoplastic aorta, coarctation of aorta, subaortic stenosis, bicuspid aortic valve, patent ductus arteriosus/ECMO</td>
<td>1 mo</td>
<td>F</td>
</tr>
<tr>
<td>13</td>
<td>Atrial septal defect, subaortic stenosis, coarctation of aorta, bicuspid aortic valve</td>
<td>2 yr</td>
<td>M</td>
</tr>
<tr>
<td>Non-hypoxic-ischemic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Microcephaly, seizures</td>
<td>1 yr</td>
<td>F</td>
</tr>
<tr>
<td>2</td>
<td>Bacterial meningitis</td>
<td>3 yr</td>
<td>F</td>
</tr>
<tr>
<td>3</td>
<td>Neu-Laxova syndrome</td>
<td>7 yr</td>
<td>F</td>
</tr>
<tr>
<td>4</td>
<td>Ornithine transcarbamylase deficiency</td>
<td>3 yr</td>
<td>F</td>
</tr>
</tbody>
</table>

Criteria for inclusion in each of the groups are given in "Materials and Methods." M, male; F, female; ECMO, extracorporeal membrane oxygenation.

Astrocytosis was also present in three of four specimens from patients with non-hypoxic-ischemic neurological disease. A sample of cerebellar tissue obtained at the time of autopsy was immediately frozen at -80°C and used for biochemical studies to determine glutamine synthetase activity.

Results were expressed as units of enzyme activity per gram of tissue (wet weight). A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of glutamic acid γ-monohydroxamate in 15 minutes under the conditions given below.

A piece of cerebellar hemisphere (0.1–0.4 g) was removed with a scalpel from tissue previously maintained at -80°C. The sample was rapidly weighed and homogenized in 2 ml 0.15 M potassium chloride containing 10 mM β-mercaptoethanol and 2 mM EDTA at 4°C. All subsequent preparative steps were performed at 4°C. The homogenate was centrifuged at 16,000g for 30 minutes. A 50-μl aliquot of the supernatant was used to assay for glutamine synthetase activity by the method of Meister.1 Briefly, the supernatant was added to a mixture (final volume 0.55 ml) containing 0.1 M imidazole-HCl, 25 mM β-mercaptoethanol, 50 mM sodium L-glutamate, 10 mM NaATP, 20 mM magnesium chloride, and 125 mM hydroxylamine adjusted to pH 7.2 with sodium hydroxide. Blanks consisting of all assay components except ATP were prepared for each sample. This mixture was incubated for 15 minutes at 37°C. The samples were then cooled to 4°C and 0.75 ml of a cold solution containing 0.37 M ferric chloride, 0.67 M hydrochloric acid, and 0.20 M trichloroacetic acid were added. The samples were then centrifuged at 3,000 rpm and 4°C for 10 minutes. The OD of each supernatant (sample-blank) was determined at room temperature using a double-beam spectrophotometer. All analyses were performed in duplicate. Colorimetric analysis of a standard solution of glutamic acid γ-monohydroxamate (Sigma Chemical Corp., St. Louis, Mo.) after derivatization with our ferric chloride solution indicated that the variability of the assay from day to day was in the range of ±10%. Similarly, analysis of multiple pieces of cerebellar hemisphere from the same specimens revealed that the regional variation in glutamine synthetase was 6–8%. On any given day, samples from at least two of the groups were examined and in no instance were all of the members of a given group analyzed on a single day.

Results

Case Histories

Acute Patient 1: A 9-year-old male had been taking desipramine since a suicide attempt 6 months before his death. Three days before his death, he sustained a cardiorespiratory arrest after running
FIGURE 1. Glutamine synthetase activity (units/g wet tissue wt) of the cerebells of 20 autopsied patients who died with acute or chronic hypoxic-ischemic (HI) disease or with non-HI neurological disease. Results are displayed in rank order.

Chronic Patient 1: A 5-year-old female was well until 5 months before her death when she developed generalized muscle weakness, skin rash, and arthralgia. She was treated with corticosteroids for presumed dermatomyositis. One month before her death, she developed a dry cough that progressed to frank respiratory distress and cyanosis. A muscle biopsy at this time showed no evidence of inflammation and slight predominance of type I fibers. She died after 21 days of extracorporeal membrane oxygenation and corticosteroid treatment.

Non-hypoxic-ischemic Patient 1: A 1-year-old female weighed 2,180 g and had a head circumference of 30 cm (<5th percentile for age) at birth. She had Apgar scores of 9 at 1 minute and 9 at 5 minutes. Her psychomotor development was globally, severely, and consistently delayed, and she began having infantile spasms at 4 months of age; the latter evolved into a mixed seizure disorder. Her facies were dysmorphic, with upturned nares, short lips, and a flattened nasal bridge. At a chronic care facility, she was found dead in bed one morning. At autopsy, she was seen to have microcephaly with polymicrogyria and white matter hypoplasia accompanied by diffuse astrocytosis and microcalcifications.

Figure 1 shows glutamine synthetase activity levels ranked in increasing order for ease of depiction. Even without statistical confirmation, it is obvious that samples from patients who sustained acute hypoxic-ischemic insults cluster at the high end of the glutamine synthetase activity spectrum (mean 6.5, range 5.4–7.2 units/g tissue). Statistical analysis by the Kruskal-Wallis one-way analysis of variance by ranks test indicates that the three data groups do not come from one population (p<0.05). Although the sample size is too small to perform more meaningful tests to determine which of the three groups differs from the others, inspection of the data indicates that, with the exception of one "outlier," all of the values for patients with acute hypoxic-ischemic insults lie above those for patients with chronic hypoxic-ischemic insults (mean 2.6, range 1.3–3.9 units/g tissue) or neurological disease without hypoxic-ischemic insult (mean 2.8, range 0.7–10.2 units/g tissue). Glutamine synthetase activity values for patients with chronic hypoxia-ischemia overlap with those for patients with non-hypoxic-ischemic neurological disease.

Figure 2 shows that there was no correlation between the glutamine synthetase activity and the age (panel A)
Discussion

One major role played by glia in the central nervous system is the maintenance of an appropriate extracellular milieu for neuronal function. The detoxification of glutamate by glutamine synthetase in astrocytes and, perhaps, oligodendroglia, is an important homeostatic process. Although many recent studies have focused on the effects of hypoxia and ischemia on the brain glutamate concentration, little is known about the effects of these conditions on glutamate metabolism. In addition, the compensatory mechanisms of the brain in response to hypoxia-ischemia are poorly understood.

The present study demonstrates that glutamine synthetase activity is increased in the cerebellum of children who have died of acute hypoxic-ischemic injury relative to those of children who sustained chronic hypoxia-ischemia or other chronic neurological insults. Our finding of comparable degrees of astrocytosis in patients with these conditions suggests that this increase in activity is not due simply to an increase in the number of glutamine synthetase-containing cells, but rather to an induction of enzyme synthesis, an increase in enzyme half-life, or an intrinsic molecular change in the enzyme itself. It is possible as well that the severity of the hypoxic-ischemic insult was greater in the patients who died acutely than in those who tolerated a chronic insult.

The finding of comparable levels of glutamine synthetase activity in patients with non-hypoxic-ischemic disease and those with chronic hypoxia-ischemia, and the absence of "intermediate levels" in the latter group, perhaps argues against this possibility and in favor of the existence of a different compensatory mechanism in the chronic situation.

Post-hypoxic-ischemic edema is not likely to be a confounding variable in the studies we have presented. Pathological evidence for edema was found only in those specimens from patients with acute hypoxic-ischemic insults. This edema would be expected to artifactually lower enzyme activity determinations expressed per gram of wet tissue weight. Our finding of elevated enzyme activity in these specimens is made all the more striking by this fact and may therefore quantitatively underestimate the degree of elevation of glutamine synthetase activity under these conditions. Similarly, reperfusion after ischemia has been reported to decrease glutamine synthetase activity in an animal model of cerebral ischemia. The patients who were aggressively resuscitated in our study were those in the acute hypoxia-ischemia group, and glutamine synthetase activity was elevated in this group. This makes it unlikely that the aggressiveness or rapidity of resuscitation is responsible for the glutamine synthetase activity changes we report.

Petito et al have demonstrated that acute brain ischemia in rats results in an increase in glutamine synthetase activity and the number of glutamine synthetase immunoreactive cells in the cortex, striatum, and cerebellum. Our results are consistent with these findings, but also suggest that astrocytosis alone does not necessarily obligate a significant increase in glutamine synthetase activity.

The concentration of glutamate in the cerebellum is fivefold to sixfold higher than the Kon of the enzyme for this amino acid. The enzyme is therefore likely to be near-saturatied under physiological conditions, and a twofold to threefold difference in enzyme activity could therefore be of physiological as well as statistical significance.

Many other biochemical modulations have also been associated with changes in glutamine synthetase activity. Chronic hyperammonemia and corticosterone exposure lead to an increase in brain glutamine synthetase activity. However, our patient with hyperammonemia (maximum serum ammonia 525 \(\mu\)M) due to ornithine transcarbamylase deficiency and the chronically stressed patients in our study all had glutamine synthetase activity levels well below those of patients who sustained acute hypoxia-ischemia. Acute or short-term (1 week) ethanol administration, whether to cultured astrocytes or in an animal model, leads to a decrease in brain glutamine synthetase activity. The effects of chlorpromazine and insulin on glutamine synthetase activity vary markedly depending on the species and age of the animal from which cells are obtained and on the presence or absence of serum in the culture medium.

Our studies indicate that there was no significant correlation between glutamine synthetase activity and the number of hours between death and postmortem examination. Consistent with this finding, Bhargava and Telang have reported that there is no change in brain glutamine synthetase activity over at least 12 hours after death. We have also found no correlation of sex or age with glutamine synthetase activity. Interestingly, although corticosterone markedly advances the induction of glutamine synthetase activity during ontogeny, neither testosterone, estradiol, nor progesterone are inducers of this enzyme.

These studies raise two major hypotheses that are amenable to direct testing in animal and tissue culture model systems; namely, that the increase in glutamine synthetase comes in response to an increase in extracellular glutamate, and that the increase in glutamine synthetase is the result of upregulation at the transcriptional level. Newly developed technology for in vivo microdialysis could be used in animal models to measure extracellular concentrations of glutamate in specific local areas. The development of cDNA probes for...
mammalian glutamine synthetase mRNA will facilitate the testing for evidence of transcriptional upregulation of glutamine synthetase. From a clinical standpoint, it remains to be seen whether the changes we have observed in children can be generalized to adult populations. Furthermore, animal studies will help to distinguish the individual roles of hypoxia and ischemia in the mediation of increases in glutamine synthetase. Such studies will further our understanding of endogenous compensatory mechanisms and may pave the way for pharmacological interventions that might exploit such mechanisms. It is hoped that our studies, performed in the course of examining the role of glutamine synthetase in other diseases such as astrocytic neoplasia, will serve as an impetus for laboratories involved in examining biochemical changes in response to hypoxia and ischemia to confirm and further elucidate the mechanism of this phenomenon.

References


KEY WORDS • anoxia • cerebral ischemia • glutamine synthetase
Cerebellar glutamine synthetase in children after hypoxia or ischemia.
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Stroke. 1991;22:1312-1316
doi: 10.1161/01.STR.22.10.1312
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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