Differences in Ischemia-Induced Accumulation of Amino Acids in the Cat Cortex

Nobumitsu Shimada, MD, Rudolf Graf, PhD, Gerd Rosner, PhD, and Wolf-Dieter Heiss, MD

It is well established that excitatory amino acid neurotransmitters are extensively liberated during ischemia and that they have neurotoxic properties contributing to neuronal injury. To study changes in the liberation of excitatory and other amino acids during cerebral ischemia, we measured their extracellular concentrations and related them to blood flow levels and electrophysiologic activity (electrocorticogram and auditory evoked potentials) before and for up to 2 hours after multiple cerebral vessel occlusion in 14 anesthetized cats. Blood flow levels between 0 and 43 mL/100 g/min were reached. Concentrations of the excitatory amino acid neurotransmitters increased most (aspartate 10-fold, glutamate 30-fold, and \( \gamma \)-aminobutyric acid 300-fold compared with control values) below a blood flow threshold of 20 mL/100 g/min. The total power of the electrocorticogram and the amplitude of the auditory evoked potentials were affected below the same blood flow threshold. In contrast, concentrations of the nontransmitter amino acids taurine, alanine, asparagine, serine, and glutamine increased 1.5-5-fold as blood flow decreased, while concentrations of the essential amino acids phenylalanine, valine, leucine, and isoleucine did not change during cerebral ischemia. The great increases in concentrations of the excitatory amino acid neurotransmitters below a blood flow threshold close to that for functional disturbance is in accordance with the role of these amino acids in ischemic cell damage. Their release at blood flow levels compatible with cell survival and the increase in their concentrations with severity and duration of cerebral ischemia imply that excitotoxic antagonists may have potential as therapeutic agents. (Stroke 1990;21:1445-1451)

In vitro studies on brain slices and cell cultures and in vivo investigations applying intracerebral microdialysis have shown that excitatory amino acid (EAA) neurotransmitters, especially L-glutamate (Glu), are extensively liberated during hypoxia and ischemia. By their activating effect on kainate receptors, EAAs induce ionic changes, with excessive entry of \( Na^+ \), \( Cl^- \), and water and exit of \( K^+ \) leading to early cell injury by osmolysis. Additionally, delayed cell death can be triggered by \( Ca^{2+} \) influx primarily through the action of EAAs on the \( N \)-methyl-D-aspartate (NMDA) receptor-operated calcium channel and through voltage-dependent calcium channels opened during depolarization. Together with the subsequent cascade of pathophysiological events, “excitotoxicity” is postulated as a new mechanism of cell damage in ischemia and epilepsy and as indicating the potential of therapeutic strategies through the inhibition of EAA effects.

While microdialysis has demonstrated increases in the concentrations of EAA and inhibitory amino acid neurotransmitters, dopamine, and adenosine in the hippocampus and basal ganglia, studies on changes in the concentrations of amino acids in the cerebral cortex during ischemia are scarce and correlations of such changes with the severity of cerebral ischemia and functional impairment have not been obtained. In a preliminary study, we established a threshold-like relation between the severity of cerebral ischemia and the accumulation of extracellular Glu; the cerebral blood flow (CBF) level below which extracellular Glu accumulates (20 mL/100 g/min) corresponds well with the CBF threshold for impairment of auditory evoked potentials (AEFs). In the present study, we compared changes in the concentrations of other EAA neurotransmitters and nontransmitter amino acids with CBF decreases and electrophysiologic impairment.
FIGURE 1. Exposure of right subclavian artery (SC) and its branches (vertebral artery [1], costocervical trunk [2], superficial cervical artery [3], and internal thoracic artery [4]) through transverse skin incision along upper margins of pectoral muscles in cat.

Materials and Methods

We used 14 adult cats of either sex weighing 2.5–5.2 kg. Anesthesia was induced with 25 mg/kg i.m. ketamine hydrochloride. After catheterization of the left femoral artery and vein, the cats were tracheotomized, immobilized with 0.2 mg/kg i.v. pancuronium bromide, and ventilated artificially. To avoid the protective effect of ketamine against excitotoxic and ischemic damage,\(^\text{17}\) anesthesia was continued with 0.8–1.5% halothane in a 70%:30% N\(_2\)O:O\(_2\) gas mixture. Intravenous infusion of 2 ml/kg/hr Ringer’s solution containing 5 mg/kg/hr gallamine triethiodide for muscle relaxation was maintained throughout the experiment. Arterial blood pressure was monitored continuously; arterial blood gases were monitored intermittently. Paco\(_2\) was kept between 30 and 35 mm Hg. Body temperature, measured with a rectal probe, was kept at 37.5–38.5° C by means of a heating blanket.

Both common carotid arteries were exposed proximal to the origin of the superior thyroid artery near the level of the thyroid gland. The vertebral arteries, the superficial cervical arteries, and the costocervical trunks (Figure 1) were approached through a transverse skin incision along the upper margins of the pectoral muscles.\(^\text{18}\) Then 3-0 monofilament nylon sutures were placed around each exposed artery and passed through Silastic tubes to form snare ligatures.

Craniotomies were stereotactically performed over the middle ectosylvian gyrus (primary auditory cortex), and small dural and arachnoidal incisions were made, avoiding large cortical vessels. Under microscopic control, a probe/electrode assembly comprising a microdialysis probe (Carnegie Medicin, Stockholm, Sweden; tubular dialysis membrane 1 mm long and 0.5 mm in outer diameter, molecular weight cut-off of 20,000) and a macroelectrode (etched platinum/iridium wire 250 µm in diameter, glass-insulated to within 1 mm of the tip) with a tip-to-tip distance of 1 mm was then gently inserted 1.5 mm into the auditory cortex. The macroelectrode measured H\(_2\) clearance, and CBF was calculated from the initial 2 minutes of the clearance curve as 69.3×T\(_{1/2}\), where T\(_{1/2}\) is the half-time. The spontaneous electrocorticogram (ECoG) and the AEPs elicited by click stimulation were recorded; the ECoG power spectra were analyzed as the square roots of the spectral power Fourier coefficients in the range 1–20 Hz and the difference in amplitude between the first positive and the first large negative peaks of the AEP was evaluated as the primary cortical response.

The microdialysis probe was continuously perfused with Krebs-Ringer solution (122 mM NaCl, 3 mM KCl, 1.2 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 25 mM NaHCO\(_3\), and 0.4 mM KH\(_2\)PO\(_4\); pH 7.4) at a constant flow rate of 2 µl/min using a microinfusion pump (Carnegie Medicin). The concentrations of amino acids in the dialysate were analyzed by high-performance liquid chromatography (HPLC) after precolumn derivatization with o-phthaldialdehyde (OPA) according to the methods of Lindroth and Mopper.\(^\text{19}\) In brief, 10-µl aliquots of dialysate were reacted with the same volume of OPA reagent (67.1 mg OPA dissolved in 1.0 ml methanol:mercaptoethanol [20:1 vol:vol] and diluted to 10 ml with 0.4 M borate buffer [pH 9.5]). After 2 minutes, 20 µl of the derivatives was injected into an HPLC system (Knauer, West Berlin, F.R.G.). Amino acids in the dialysate were identified and quantified by comparing retention times and peak areas with those of external standards.

Steady-state levels of CBF and extracellular amino acid concentrations were reached 2 hours after probe/electrode assembly implantation. Thereafter, dialysate was collected at 10-minute intervals. Following 30 minutes of control measurements in all 14 cats, mild to moderate brain ischemia was induced in nine cats by occluding the common carotid and vertebral arteries for 2 hours. To generate more severe ischemia in the other five cats, the superficial
cervical arteries and the costocervical trunks were also occluded for 2 hours. After vascular occlusion systemic blood pressure increased from the control level of 111.7±21.9 mm Hg (mean±SD, n=14) to 156.9±40.5 mm Hg and then gradually returned to the control value within approximately 1 hour.

**Results**

During the control period, mean±SD CBF was 55.8±10.7 ml/100 g/min (n=14) in the auditory cortex. After vascular occlusion, CBF fell immediately and (depending on the cat and on the extent of occlusion) individual CBF values ranged from 0 (no saturation of H2) to 43 ml/100 g/min (Figure 2). Within a cat, CBF was maintained at a fairly constant level during the 2 hours of ischemia.

Mean±SEM extracellular concentrations of 12 amino acids in the dialysate during the control period are listed in Table 1. Changes in the extracellular concentrations of Glu, γ-aminobutyric acid (GABA), taurine (Tau), and leucine (Leu) for each cat during the 2 hours of ischemia are illustrated in Figure 3. Depending on the severity of ischemia, the concentrations of Glu and GABA increased by up to 30-fold and 300-fold the control value, respectively, while the concentration of Tau increased by only about fivefold and that of Leu remained nearly unchanged. The relations between CBF reduction and extracellular concentrations of the 12 amino acids 50-60 minutes after occlusion are plotted for each cat in Figure 4. A threshold-type relation was obtained for the EAA neurotransmitters aspartate and Glu and the inhibitory neurotransmitter GABA, with sharp increases in their concentrations when CBF dropped below approximately 20 ml/100 g/min. Nonessential amino acids not considered transmitters in the cortex (Tau, alanine, asparagine, serine, and glutamine) showed less marked alterations in concentrations during ischemia (1.5-5-fold), and no threshold-type relation between CBF and concentration was found. The extracellular concentrations of other essential amino acids (phenylalanine, valine, isoleucine, and Leu) were not related to CBF reduction and did not change during ischemia.

The changes in ECoG power and AEP amplitude during ischemia resembled those of extracellular EAA neurotransmitter concentration. Electrical impairment started at the same CBF threshold as EAA neurotransmitter accumulation (Figure 5).

**Discussion**

To study the dependency of amino acid liberation on CBF, a model that permits various levels of graded ischemia ranging from slightly below normal to cessation of perfusion is required. We achieved this prerequisite by occluding the major brain vessels

| TABLE 1. Extracellular Concentrations of Amino Acids in Dialysate From Cat Auditory Cortex Before Cerebral Ischemia |
|-----------------|-----------------|
| **Amino acid**  | **Concentration** |
|                 | (μmol/l)        |
| Aspartate       | 0.30±0.03       |
| Glutamate       | 0.96±0.12       |
| Asparagine      | 0.16±0.03       |
| Serine          | 0.86±0.11       |
| Glutamine       | 9.92±0.81       |
| Taurine         | 1.29±0.17       |
| Alanine         | 1.04±0.18       |
| α-Aminobutyric acid | 0.02±0.003  |
| Valine          | 0.97±0.10       |
| Phenylalanine   | 0.39±0.04       |
| Isoleucine      | 0.40±0.04       |
| Leucine         | 0.69±0.09       |

Values are mean±SEM, n=14.
The onset of increases in the extracellular concentrations of amino acids depends on the severity of cerebral ischemia; complete global ischemia induces 4–8-fold increases in the concentrations of Glu and aspartate after 10 minutes and >100-fold increases after 30 minutes, but the increases differ for the various amino acids. This can be seen in Figure 3, where significant changes are obvious in the cats with the most severe ischemia for Glu after 10, for GABA after 20, and for Tau after 30 minutes. As described previously, the extracellular accumulation of amino acids increases with time, reaching maximal values after 60 minutes. To obtain maximal effects of mild and moderate ischemia but not lose the effects on nontransmitter amino acids, we used the extracellular concentrations after 50–60 minutes of ischemia to establish the relation with severity of ischemia. Using this approach, different behaviors of the various amino acids could be described, with a steep increase in the concentrations of neurotransmitters below a threshold of approximately 20 ml/100 g/min, a slight increase in the concentrations of nonessential amino acids (some with possible modulator action), and no increase in concentrations of amino acids essential for protein synthesis. This selective increase in the concentrations of EAA neurotransmitters near the CBF threshold for electrophysiologic function indicates the liberation of these amino acids from nerve terminals due to depolarization of nerve cells and forms the basis of excitotoxicity in the brain cortex. EAAs can trigger early cell death by activating the kainate receptors that gate sodium channels and promote Cl− and water uptake, leading to osmolysis; these kainate receptors are abundant on pyramidal cells. Membrane depolarization caused by influx of Na+ might open voltage-dependent calcium channels, leading to an influx of Ca2+. The NMDA receptor also gates a calcium channel. Therefore, EAAs open two pathways by which Ca2+ influx mediates delayed neuronal injury. The selective vulnerability of nerve cells depends on the distribution of NMDA receptors, which might be related to the occurrence of seizure-like activity during ischemia-induced Ca2+ accumulation. Seizure-like activity is a common finding in the hippocampus of ischemic gerbils, but it has also been demonstrated in the cortex of cats during ischemia.
and early reperfusion, and epileptic seizures are repeatedly documented in patients with acute ischemic cerebrovascular disease.

While the importance of EAAs for ischemic neuronal damage is well established and supported by our finding increases in their concentrations at CBF levels below the threshold for neuronal function and synaptic transmission, the extracellular accumulation of the inhibitory neurotransmitter GABA, which should protect against excitability-induced cell injury, needs further discussion. That the increased concentration of GABA, which is liberated from depolarized inhibitory interneurons, does not protect against or at least ameliorate neuronal damage might be due to the fact that, contrary to the Glu receptors (which are resistant to ischemia), the functioning of GABAergic receptors is impaired in ischemia. This might also explain why GABA agonists are not very effective in preventing ischemic cell damage. Whether graded CBF-related increases in concentrations of Tau, aspartate, serine, and glutamine (of which at least Tau has a modulatory property in regulating osmolality of the brain) affect ischemic cell damage has not been investigated.

We conclude that EAAs are released at CBF values close to the thresholds for neuronal function.
and synaptic transmission, consistently found to be 12–18 ml/100 g/min (see Reference 36). With more severe and longer-lasting ischemia, the extracellular concentrations of EAAs increase further since release continues and neuronal and glial reuptake is impaired.13 This explains why extremely high concentrations are reached after 50–60 minutes of severe cerebral ischemia. This gradual increase in the concentrations of EAAs starting at CBF values above the functional threshold and becoming more severe with the duration of ischemia (which corresponds to the time window of morphologic damage to neurons during ischemia27) leaves some hope for therapeutic strategies. However, antagonists of EAA neurotransmitters,10,11 as well as drugs blocking Ca2+ entry,37,38 must be applied early after the onset of cerebral ischemia to protect against continuing neuronal damage and thereby ameliorate the clinical course and outcome after stroke.

References
minute ischemia in Mongolian gerbils: II. Changes of sponta-
neous neuronal activity in cerebral cortex and CA1 sector of
hippocampus. Acta Neuropathol (Berl) 1983;60:217–222
26. Heiss WD, Hayakawa T, Waltz AG: Cortical neuronal func-
tion during ischemia—Effects of occlusion of one middle
cerebral artery on single-unit activity in cats. Arch Neurol
1976;33:813–820
27. Heiss WD, Rosner G: Functional recovery of cortical neurons
as related to degree and duration of ischemia. Ann Neurol
1983;14:294–301
28. Louis S, McDowell F: Epileptic seizures in nonembolic cere-
29. Barolli GS, Scherzer E, Schnaberth G: Die zerebrovaskular-
bedingten Anfälle. Bern, Hans Huber, 1975
30. Cocito L, Favale E, Reni L: Epileptic seizures in cerebral
31. Westerberg E, Monaghan DT, Cotman CW, Wieloch T: Ex-
citatory amino acid receptors and ischemic brain damage in
32. Dewar D, Wallace MC, Kurumaji A, McCulloch J: Alterations
in the N-methyl-o-aspartate receptor complex following focal
33. Schwartz RD, Skolnick P, Steven MP: Regulation of gamma-
aminobutyric acid/barbiturate receptor-gated chloride ion flux
in brain vesicles by phospholipase A2: Possible role of oxygen
34. Sternau LL, Lust WD, Ricci AJ, Ratcheson R: Role for γ-
aminobutyric acid in selective vulnerability in gerbils. Stroke
1989;20:281–287
35. Wade JH, Olson JP, Samson FE, Nelson SR, Pazdernik TL: A
possible role for taurine in osmoregulation within the brain. J
Neurochem 1988;51:740–745
36. Heiss WD: Progress in cerebrovascular disease: Flow thresh-
olds for functional and morphological damage of brain tissue.
Stroke 1983;14:329–331
37. Godfraind P, Miller R, Wibo M: Calcium antagonism and
38. Choi DW: Calcium-mediated neurotoxicity: Relationship to
specific channel types and role in ischemic damage. Trends
Neurosci 1988;11:465–469

KEY WORDS cerebral blood flow • neurotransmitters •
aminos acids • cats
Differences in ischemia-induced accumulation of amino acids in the cat cortex.
N Shimada, R Graf, G Rosner and W D Heiss

*Stroke*. 1990;21:1445-1451
doi: 10.1161/01.STR.21.10.1445

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 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:
http://stroke.ahajournals.org/content/21/10/1445

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/