Effect of Autologous Clot Embolism on Regional Protein Biosynthesis of Monkey Brain

Y. Xie, MD, K. Munekata, MD, K. Seo, MD, and K.-A. Hossmann, MD, PhD

Progress in understanding the pathophysiology of experimental brain ischemia has led to an increasing interest in the early detection and evaluation of cerebrovascular accidents under clinical conditions. Various noninvasive imaging techniques that allow the visualization of hemodynamic, metabolic, and structural abnormalities have become available for this purpose. Analysis of these data and comparison with experimental studies have revealed a close correlation between metabolic disturbances and structural defects after manifestation of irreversible ischemic injury. However, considerable dissociations between the various parameters may be present during the acute phase of infarct development. In particular, reperfusion of the ischemic territory by collateral blood supply or after reversal of the vascular obstruction, stimulation of anaerobic glycolysis at reduced blood flow rates, or uncoupling of oxidative phosphorylation may provide images of high blood flow or metabolic rate although the tissue has been irreversibly damaged by the primary ischemic insult. In fact, when images of blood flow and glucose utilization were compared with the distribution of metabolites after 2 hours of middle cerebral artery occlusion in cats, considerable dissociations between both the severity and the spatial distribution of pathologic alterations became apparent. It was particularly confusing that circumscribed regions with normal or enhanced blood flow and reduced protein synthesis, might be better suited to visualization of the injured territory.

Protein synthesis is an endergonic metabolic pathway that is suppressed as soon as energy stores of the brain are depleted. Following a transient period of ischemia, protein synthesis is not immediately resumed because ribosomes disaggregate upon recirculation and, depending on the duration of ischemia, may remain in a disaggregated state for many hours. This leads to a prolonged state of impaired protein synthesis even if energy metabolism is resumed. The demonstration of depressed protein synthesis, therefore, may be useful after a period of ischemia to identify those regions that have suffered a significant metabolic injury. Persisting depression of protein synthesis has also been shown to precede delayed postischemic neuronal death and, therefore, may be a valuable predictor of selective neuronal injury.

In our study the potential of imaging disturbances in amino acid incorporation into brain proteins has been studied following experimental embolism of the monkey brain with autologous clots. We used the tracer [1H]phenylalanine because an analogue of this amino acid, [18F]fluorophenylalanine, is accepted by phenylalanine-tRNA-synthetase and thus may be suitable for clinical imaging by positron emission tomography.

Materials and Methods

General Preparation and Clot Embolism

Six adult monkeys of either sex, weighing 3-7 kg, were used. Two monkeys were sham-operated controls, and four monkeys were used for autologous clot embolization of the brain (experimental Animals A-D). In the experimental monkeys, 2-3 ml venous blood were taken 2 days before the experiment and allowed to coagulate at room temperature. The clots were separated from serum and passed three times through an 18-gauge hypodermic needle to form cylinders of approximately 1 mm diameter. These cylinders were cut into 5-7 mm-long fragments and suspended in 4°C Ringer’s solution until use.

On the day of embolization, monkeys were anesthetized with 5 mg/kg i.m. ketamine followed by
continuous intravenous infusion at 2 mg/kg/hr, tracheotomized, immobilized with pancuronium, and artificially ventilated with a mixture of 30% oxygen and 70% nitrous oxide. Paco, was adjusted to about 45 mm Hg by appropriate setting of the tidal volume of the respirator. Body temperature was kept constant at 36.5°C with a feedback-controlled heating pad. Catheters were inserted into a femoral artery and a femoral vein for recording of arterial blood pressure, for blood sampling, and for injection of drugs.

The bifurcation of the left common carotid artery was exposed through a midline neck incision, and the internal and external carotid arteries were identified. The external carotid artery was ligated, and the internal carotid artery was punctured nonocclusively with a sharp needle connected to a polyethylene catheter for injection of clots. After stabilization of physiologic parameters, embolization was carried out by flushing the blood clots suspended in Ringer's solution into the carotid artery. Each of the four experimental monkeys received five clot emboli at 1-minute intervals; the control monkeys were injected with Ringer's solution.

Two hours after embolization, monkeys received a bolus of [3H]phenylalanine for measurement of protein synthesis (see below); 45 minutes later cardiac arrest was induced by intravenous injection of 1 ml saturated potassium chloride. Craniotomy was rapidly performed, and a 1-cm horizontal slice passing through the parietal lobes was sampled and frozen in butanol precooled to −50°C. Immediately after removal of this slice, the brain was fixed in situ by transcardiac perfusion with 4% buffered formaldehyde.

Electrophysiologic Recordings
Silver-plated needle electrodes were inserted bilaterally into the scalp at the level of the frontal, temporal, and occipital lobe. Bipolar electroencephalographic (EEG) recordings were registered on a polygraph and stored on magnetic tape for off-line frequency analysis. Fourier transformation was carried out with a laboratory computer (PDP-12, Digital Equipment Corp., Maynard, Massachusetts), and the magnitude of EEG intensity was determined by summing the square roots of Fourier coefficients over the spectral range from 0.5 to 20 cps.

Somatosensory evoked potentials were recorded from the frontotemporal electrodes after electrical stimulation of the contralateral forepaw with square-wave pulses of 0.3 msec duration and 5 V intensity. Fifty responses were averaged with a biomedical signal processor (MED 80, Nicolet Instruments Corp., Madison, Wisconsin), and alterations were expressed as percent changes of the peak-to-peak amplitudes.

Measurement of Protein Synthesis
Protein synthesis was evaluated by measuring time-integrated incorporation of [3H]phenylalanine into brain proteins. The tracer (1 mCi/kg, specific activity 47 Ci/mmol) was injected into a femoral vein 2 hours after embolization. During the 45-minute incorporation period, seven 0.2-ml arterial blood samples were taken at increasing time intervals for determination of the input function of the blood precursor pool. Samples were immediately centrifuged, and sera were stored at −80°C until use. After termination of amino acid incorporation (i.e., 2 hours and 45 minutes after embolization), brain material was processed for quantitative autoradiography and biochemical analysis.

 Autoradiography and Histology
The formalin-fixed brain was cut into four horizontal slices, dehydrated, and embedded in paraffin. Brain sections of 8 μm thickness were prepared and arranged in a x-ray film cassette. Autoradiography was carried out by exposure of these sections to 3H-sensitive film (Ultralum, LKB Produkter AB, Stockholm, Sweden) for 21 days. Alternate sections were stained with hematoxylin and eosin and cresyl violet and then processed for routine light microscopy.

Biochemical Analysis
Brain samples (5–10 mg) taken from the frozen parietal lobe were homogenized at 4°C in distilled water by sonication. An equal volume of 10% trichloroacetic acid (TCA) was added to the homogenate, and the samples were kept at 4°C for 1 hour. TCA-soluble and -insoluble components were separated by centrifugation. The pellets were washed twice with 5% TCA and dissolved in 2% Na2CO3. Aliquots of supernatants and dissolved pellets were taken for measurement of radioactivity using a liquid scintillation counter (LS 7000, Beckman Instruments Inc., Fullerton, California). The protein content was determined according to Lowry et al.18

The TCA-soluble supernatants were used for high-performance liquid chromatography analysis of amino acids. The chromatographic system consisted of a M 720 system controller, two A-6000 pumps, a WISP 710B automatic sample injector, and a Model 400 UV detector (Waters Chromatography Division, Eschborn, F.R.G.). Samples were extracted five times with diethyl ether for removal of TCA. Precolumn derivatization was carried out with dansyl chloride according to Badoud and Pratz.19 Amino acids were analyzed on a reversed-phase column (Brownlee RP-300, 250 × 4 mm, Kontron Instrument, Eching, F.R.G.) at 55°C. The mobile phase was a gradient of 10–85% 2-isopropanol in acetate buffer (pH 2.6), flow rate was 4 ml/min, and the analysis lasted 13 minutes.

Blood plasma was processed in the same way as the tissue samples. To determine whether the tissue samples were from ischemic or nonischemic regions, 20-μm cryostat sections were prepared from frozen parietal lobes for qualitative autoradiography to visualize the local amino acid incorporation.

Statistical Analysis
Differences in the magnitude of EEG intensity before and after embolization were tested by the nonparametric Wilcoxon-Mann-Whitney U test. Values are given as mean ± SD.
which affected both hemispheres to a similar degree. The only consistent observation was a global reduction in the electrophysiologic abnormalities.

**Electrophysiologic Recordings**

In the two control monkeys neither EEG nor evoked potentials changed significantly during the observation period. In the experimental monkeys, diversity of the localization of clot emboli was reflected by a heterogeneous pattern of electrophysiologic abnormalities. The only consistent observation was a global reduction of EEG amplitude immediately after embolization, which affected both hemispheres to a similar degree (Table 2). This suppression was partly reversed, but at the end of the observation period neither the amplitudes nor the frequency of EEG correlated with the severity or the localization of the ischemic injury (Figure 1).

### Results

#### General Physiologic Observations

Before embolization, all physiologic parameters were within normal limits (Table 1). Intracarotid injection of five clot emboli of 1 mm diameter and 5–7 mm length did not cause any perturbations of the general state. As demonstrated in Table 1, neither blood pressure nor any blood parameter changed during or after embolization. As a consequence, the metabolic disturbances of the brain described below are not modulated by general physiologic alterations.

#### Localization of Emboli

At the end of the experiment, the brain vascular system was carefully examined under the operating microscope for identification of clot emboli. In Animal A no emboli could be detected in the main supplying arteries of the brain. In Animal B emboli lodged at the origin of the left middle cerebral artery and in Animal C, at the origin of the right middle cerebral artery. In Animal D emboli occluded the anterior communicating artery from which both anterior cerebral arteries originate. In none of the monkeys could multiple embolization be detected macroscopically or under the operating microscope. Dissection of the brain did not reveal signs of anemic or hemorrhagic infarction.

#### Protein Synthesis

In the two control monkeys, the autoradiographic appearance of [3H]phenylalanine incorporation into brain proteins was consistent with the well-known incorporation pattern of other labeled amino acids such as valine, leucine, tyrosine, or mixtures of various amino acids. The intense labeling of neurons produced a sharp contrast between gray and white matter and allowed the precise anatomic localization of all cortical and subcortical neuronal structures (Figure 3). The biochemical assay revealed that 61% of brain radioactivity was incorporated into proteins (Table 3). The radioactivity of brain free [3H]phenylalanine accounted for only 13% of the TCA-soluble radioactivity, the remaining 87% being metabolites of the tracer.

### Table 2. Fourier Frequency Analysis of EEG in Monkeys Before and After Injection of Autologous Clot Into Left Carotid Artery

<table>
<thead>
<tr>
<th>Time after embolization</th>
<th>Left frontotemporal recording</th>
<th>Right frontotemporal recording</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Magnitude</td>
<td>Delta</td>
</tr>
<tr>
<td>Control</td>
<td>9314 ± 3754</td>
<td>57.43</td>
</tr>
<tr>
<td>Immediately</td>
<td>6789 ± 1179*</td>
<td>56.39</td>
</tr>
<tr>
<td>60 min</td>
<td>7290 ± 2397†</td>
<td>58.24</td>
</tr>
<tr>
<td>120 min</td>
<td>6687 ± 1998*</td>
<td>55.00</td>
</tr>
</tbody>
</table>

EEG magnitude as sum of square roots of Fourier coefficients from 0.25 to 20 cps. Magnitude of EEG in four frequency bands (delta 0.25–3.75, theta 4.0–7.75, alpha 8.0–12.75, beta 13.0–20.0 cps) as percent of total EEG magnitude.

*† p<0.01, p<0.05, respectively, different from control.
Following autologous clot embolization, the ischemic territories were remarkably well-demarcated by qualitative autoradiography of amino acid incorporation into proteins. Only in Animal A were lesions absent, which is in line with the macroscopic and electrophysiologic findings. In the other three experimental monkeys, circumscribed zones of sharply reduced radioactivity were present. In Animal B, in which the left middle cerebral artery was occluded, a widespread lesion was noted in the left hemisphere, involving the dorsofrontal, temporal, and parietal cortex, the white matter, and parts of the putamen and caudate nucleus. Animal C, with occlusion of the right middle cerebral artery, revealed lesions in both hemispheres; in the left hemisphere the parieto-occipital lobe and in the right hemisphere the occipital lobe were mainly affected. Animal D, with occlusion of both anterior cerebral arteries, exhibited multiple foci of reduced protein synthesis in both hemispheres; these were located mainly in the caudate nucleus, the striatum, and the parieto-occipital cortex, with dominance of the right side. A common feature of all lesions was the sharp demarcation from the surrounding normal brain tissue. Within each lesion protein synthesis was homogeneously reduced, and there were no intrafocal or perifocal foci of increased radioactivity.

The biochemical assay of tissue radioactivity in experimental monkeys did not differ much from that in the controls (Table 3). This is attributed to the fact that the dorsal part of the parietal lobe, which was sampled for biochemistry, was little affected by ischemia in this model. According to the autoradiograms, only the sample from the right hemisphere of Animal D originated from an ischemic focus with reduced amino acid incorporation. The amount of incorporated radioactivity in this sample was reduced to 60% of the control, and the protein content was also lower than in non-ischemic regions.

The integrated plasma radioactivity did not change significantly in the experimental monkeys, but the specific radioactivity of phenylalanine increased both in blood and in ischemic brain regions. This implies that the postembolic reduction of amino acid incorporation was more severe than was expressed by the relative change of the radioactivity of brain proteins.

**Histopathology**

Horizontal sections from formalin-fixed brain were stained with hematoxylin and eosin and cresyl violet and were examined by light microscopy. In Animal A histopathologic alterations were absent, but multiple ischemic lesions were detected in Animals B, C, and D. In Animal D microembolism with perifocal round cell infiltration was present in a small vessel between the left caudate nucleus and putamen. Perivascular spaces were expanded in all monkeys; in addition, neuropil of Animal B exhibited a distinct spongy state.
resulting in increased volume of the left hemisphere and slight displacement of the midline to the right.

Neuronal alterations within areas of reduced amino acid incorporation were variable (Figure 4). In Animal B the region in the left parieto-occipital cortex corresponded to a sharply demarcated coagulation necrosis with typical ischemic cell changes (ICC), characterized by shrunken, darkly stained cytoplasm and pyknotic nuclei. Other regions of reduced amino acid incorporation exhibited columnar or laminar neuronal necrosis. In Animal C the striatal areas of reduced amino acid incorporation were associated with cytoplasmic microvacuolization, scattered large neurons with homogenizing cell change, and small foci of neurons with ICC. Finally, the histopathologic pattern was almost normal in the area of reduced amino acid incorporation in the left caudate nucleus of Animal D. In this region only a few sporadic neurons with ICC were detected.

**Correlation Between Biochemical and Functional Disturbances**

The localization of the ischemic lesions by [3H]phenylalanine autoradiography correlated remarkably well with the postembolic changes of the somatosensory evoked potentials. In Animal A, in which biochemical lesions were absent, evoked potentials returned to normal within a few minutes after embolization. In Animal B, with unilateral lesions, the ipsilateral evoked potential, and in Animal C, with bilateral lesions, both the ipsilateral and the contralateral evoked potentials were suppressed. In Animal D, with frontal embolism, preferential involvement of the right hemisphere was reflected by the more pronounced disturbance of the right-sided evoked potential. It was not possible, however, to establish a metabolic correlate of the reversibility of the functional deficit. In Animals B and C a similar inhibition of protein synthesis was present in the left hemisphere, but in one monkey the left cortical evoked potential recovered while the other did not. The autoradiographic image, in consequence, correlates with the electrophysiologic suppression present shortly after embolization and not with the functional state at the time of the biochemical measurement. Amino acid incorporation into proteins, therefore, traces the initial ischemic impact of clot embolization for as long as 2 hours after the pathologic event.

**TABLE 3. **[3H]Phenylalanine Incorporation Into Brain Proteins of Monkeys After Injection of Autologous Clot Into Left Carotid Artery

<table>
<thead>
<tr>
<th></th>
<th>Brain radioactivity</th>
<th>[3H]Phenylalanine</th>
<th>Phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Total (nCi/mg)</td>
<td>TCA-precipitable (nCi/mg)</td>
</tr>
<tr>
<td>Controls</td>
<td>2</td>
<td>1.215 ±0.092</td>
<td>0.745 ±0.092</td>
</tr>
<tr>
<td>Embolization</td>
<td>4</td>
<td>1.260 ±0.402</td>
<td>0.605 ±0.245</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1</td>
<td>1.010</td>
<td>0.370</td>
</tr>
<tr>
<td>Sample 2</td>
<td>3</td>
<td>1.500 ±0.294</td>
<td>0.837 ±0.148</td>
</tr>
<tr>
<td>Sample 3</td>
<td>4</td>
<td>1.260 ±0.402</td>
<td>0.605 ±0.245</td>
</tr>
</tbody>
</table>

Cortical tissue samples from embolized monkeys were categorized according to autoradiography: Sample 1 from region with reduced amino acid incorporation; Sample 2 from border zone of focus with reduced amino acid incorporation; Sample 3 from regions with normal amino acid incorporation. Values are mean±SD.
Cerebral Protein Synthesis After Embolism

FIGURE 4. Histologic appearance of basal ganglia in areas with normal and suppressed amino acid incorporation into brain proteins. Top: control monkey. Middle and Bottom: clot embolization of left carotid artery (Animals D and B; see also Figure 3). Left: unstained autoradiogram of left hemisphere; Right: cresyl violet staining (magnification × 205) of regions indicated on corresponding autoradiograms by arrows. Note coexistence of normal and ischemic neurons in region with reduced protein synthesis (Animal D, middle) and severe ischemic cell injury in similar region (Animal B, bottom).

Discussion

Autoradiographic imaging of brain protein biosynthesis has been previously used to study metabolic recovery after transient global ischemia\textsuperscript{8,10,14} but not to localize focal ischemic disturbances. Our investigation demonstrates that protein autoradiography is equally suited for the latter purpose. In all monkeys with functional deficits after clot embolization, sharply demarcated areas of markedly reduced radioactivity that could be clearly differentiated from the surrounding brain parenchyma were present. Within the affected regions radioactivity was evenly reduced, and in the surrounding brain tissue a normal incorporation rate without any regional abnormalities was noted. This pattern is different from blood flow or glucose utilization, which exhibit greatly inhomogeneous abnormalities during the early phase of infarct development.\textsuperscript{5,6}

This difference can be readily explained on the basis of current pathophysiologic concepts of brain ischemia. Occlusion of an intracerebral artery is partly compensated for by development of collateral blood flow from the surrounding noninfarcted vasculature.\textsuperscript{20} The magnitude of collateral blood flow depends on local blood perfusion pressure and on segmental vascular resistance, which decreases at the arteriolar level because of tissue acidosis but rises in the capillaries because of increased blood viscosity and capillary compression by swollen endothelial and perivascular glial cells.\textsuperscript{20} The resulting heterogeneous blood flow pattern is associated with a heterogeneous rate of glucose utilization that is further accentuated by the stimulation of glycolysis at low blood flow rates.\textsuperscript{6}

Protein synthesis, in contrast, exhibits a threshold-dependent monophasic suppression that persists for extended periods of time even if blood flow or glucose utilization is resumed.\textsuperscript{8,10} This is due to the fact that ischemia causes ribosomal disaggregation that is only slowly reversed despite normalization of blood flow and energy metabolism.\textsuperscript{1,2} In consequence, blood flow and glucose utilization reflect the momentary state of cerebral hemodynamics and metabolism, whereas the depression of protein synthesis traces the primary ischemic impact.

Although the autoradiographic localization of areas with reduced protein synthesis was unequivocal, quantification of this change poses serious methodologic problems. The kinetics of amino acid incorporation into brain proteins following systemic application of a tracer dose are complex and difficult to evaluate.\textsuperscript{21-23} A precise measurement of the protein synthesis rate requires knowledge of the rate constants for influx and efflux of amino acids across the blood–brain barrier, specific radioactivity of the various precursor pools in the brain, and rate of protein catabolism. Some of these variables have been determined previously under physiologic conditions,\textsuperscript{24} but they cannot be readily applied to pathophysiologic states because they may be subject to changes.

The interpretation of our data, however, suggests that the errors induced by these methodologic constraints are of little practical consequence for the study of focal ischemia. Although clot embolism results in an extremely heterogeneous pattern of multifocal ischemia and although lysis of clots may cause recirculation and even hyperemia in circumscribed areas of the affected territories,\textsuperscript{25-27} we observed only decrease and never increase of amino acid incorporation into brain proteins. Measurement of the free amino acid precursor pool in brain tissue furthermore revealed that in regions with reduced amino acid incorporation, specific activity of phenylalanine was increased above normal. This demonstrates that amino acid influx is not a limiting factor and that the low radioactivity in ischemic areas of our autoradiograms does not underestimate the residual protein synthesis rate.

The possibility of tracing focal brain lesions for many hours after the ischemic insult opens interesting perspectives. The combination of amino acid autoradiography with measurements of regional cerebral blood flow and glucose utilization by double-
triple-tracer autoradiography allows the differentiation between regions of the brain that have been recirculated and have begun to recover after embolization from those in which blood flow and/or glucose metabolism has not been restored. Multiple-tracer autoradiography would also allow determination of the thresholds of ischemia, resulting in impairment of protein synthesis. A previous study suggested that reduction of synthesis may occur already at a blood flow of 50 ml/100 g/min, but more detailed investigations are necessary to establish this relation for clot embolism.

The combination of amino acid autoradiography with histology permits the precise identification of those territories that have been critically affected by the ischemic insult and thus allows differentiation between reversible and irreversible changes. Our study, in fact, was able to detect foci of normal histology in regions of therapeutic interventions under conditions of heterogeneous disturbances is not considered. Visualization of ischemic lesions by amino acid incorporation into brain proteins, as a consequence, will be mainly an experimental tool which, however, warrants further exploration.

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References

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