Ischemic Threshold of Brain Protein Synthesis After Unilateral Carotid Artery Occlusion in Gerbils

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The threshold of the relation between regional cerebral blood flow and regional cerebral protein synthesis was investigated in gerbils submitted to a 1-hour occlusion of the left common carotid artery. Blood flow was measured with [125I]iodoantipyrine and protein synthesis with [14C]leucine using double-tracer autoradiography and trichloroacetic acid wash-incubation for removal of nonincorporated tracer radioactivity. Specific activity of blood and brain leucine and [14C]leucine incorporation into brain proteins was also measured by conventional high-performance liquid chromatography to validate the autoradiographic approach. In control gerbils, gray matter blood flow ranged between 180 and 220 ml/100 g/min and fractional amino acid incorporation was approximately 80%. Unilateral carotid artery occlusion resulted in graded ischemia with blood flow between 10 and 100 ml/100 g/min. Regional cerebral protein synthesis gradually declined at blood flows of <100 ml/100 g/min and approached 0 at a blood flow of 40 ml/100 g/min. This threshold for complete suppression of protein synthesis is much higher than that for maintenance of tissue energy state and suggests that the size of an infarct after focal ischemia is determined by the suppression of protein synthesis rather than by the breakdown of energy metabolism. (Stroke 1989;20:620-626)

The effects of focal cerebral ischemia on brain function and metabolism have been studied in numerous experimental and clinical investigations. To determine the relation between the reduction in blood flow and the resulting brain injury, blood flow thresholds for the suppression of various functional and metabolic processes have been established. For example, spontaneous neuronal activity, reflected by the electroencephalogram, is already impaired when blood flow is reduced by approximately 50–60%. Decreasing cerebral blood flow to <30% of baseline leads to tissue hypoxia and results in the stimulation of anaerobic glycolysis. When blood flow further decreases, to approximately 15% of baseline, synaptic transmission is abolished. At a blood flow of <10% of baseline, energy demands of the tissue can no longer be covered, neurons depolarize, and cellular ion gradients break down due to the failure of energy-dependent ion pumps. As a result, extracellular potassium concentration rises and sodium, calcium, and chloride ions move from the extracellular into the intracellular compartment. In addition, various molecular processes are triggered that are thought to contribute to the development of ischemic cell injury (for review see Siesjo).

If residual blood flow remains above the threshold for maintenance of cell membrane function but below that for synaptic transmission, the tissue is functionally silent but intact with respect to cellular ion homeostasis and energy state ("ischemic penumbra"). Although the pathophysiologic concept of the ischemic penumbra is generally acknowledged, it remains unclear if tissue can survive in this state for extended periods. In particular, it has not been established if preservation of the energy state is equivalent to the maintenance of more complex metabolic functions such as protein or phospholipid metabolism. Persistent inhibition of protein synthesis has, in fact, been observed after transient cerebral ischemia in the vulnerable CA1 hippocampal subregion despite the rapid normalization of energy metabolism after recirculation. This fact implies that it is not the energy failure per se, but the prolonged disturbance of protein biosynthesis that is responsible for the manifestation of delayed postischemic neuronal death.

Similar dissociations between energy metabolism and protein synthesis may also occur in brain...
regions with focally reduced blood flow if the thresholds for the various metabolic pathways are different. To investigate this question, we have determined the relation between regional cerebral blood flow and regional cerebral protein synthesis in a model of graded focal ischemia, using double-tracer autoradiography.

**Materials and Methods**

We used eight male gerbils (*Meriones unguiculatus*) weighing 50–80 g. The gerbils were anesthetized with 1.2% halothane delivered in a gas mixture of 30% oxygen and 70% nitrogen. Body temperature was maintained at 37°C by means of a feedback-controlled infrared lamp. The left common carotid artery was exposed through a midline neck incision and carefully dissected from connective tissue and the vagus nerve. In two control gerbils, surgery was terminated at this point and the skin incision was sutured. In six ischemic gerbils, the carotid artery was double-ligated with 5-0 silk and cut between the ligatures. Thirty minutes later in both groups of gerbils, catheters were inserted into both femoral arteries and one femoral vein for measurement of blood pressure, withdrawal of arterial blood samples, and intravenous infusions. Radioactive tracers for the measurement of cerebral blood flow and cerebral protein synthesis were infused as described below. As soon as appropriate incorporation times had elapsed, the gerbils were decapitated and the brains were rapidly removed from the skulls and frozen at −50°C in precooled isopentane.

One hour after surgery, the tracer L-[1-14C]leucine (100 μCi, specific activity 54.4 mCi/mmol; New England Nuclear, Dreieich, FRG) was injected intravenously during 30 seconds for the measurement of cerebral blood flow and cerebral protein synthesis were infused as described below. As soon as appropriate incorporation times had elapsed, the gerbils were decapitated and the brains were rapidly removed from the skulls and frozen at −50°C in precooled isopentane.

Thirty minutes after the injection of [14C]leucine (i.e., 1 hour and 30 minutes after surgery) cerebral blood flow was measured with the tracer [131I]-iodoantipyrine (IAP, 100 μCi, specific activity 7.45 mCi/mg; Amersham, Braunschweig, FRG) applied intravenously by ramp infusion over 30 seconds. Thereafter, blood flow measurements were terminated by decapitation. During the infusion, arterial blood samples were collected from a short, freely flowing arterial catheter onto preweighed filter paper. Iodine-131 radioactivity of the arterial blood was measured in a gamma counter (Biogamma II, Beckman Instruments, Inc., Fullerton, California) and expressed as nanocuries per milliliter.

Within 24 hours after the experiment, the frozen brains were transferred over liquid nitrogen to a cryostat microtome (SLEE, Mainz, FRG); 20-μm coronal cryostat sections were prepared from 0.2 mm rostral to 2 mm caudal to the bregma and immediately dried on a hot plate at 60°C to prevent diffusion of the IAP. In addition, tissue samples were excised from the frontal and parietal cortices of both hemispheres for later quantitative analysis of tissue carbon-14 radioactivity.

Double-tracer autoradiography of carbon-14 and iodine-131 activity was carried out by taking advantage of the different half-lives of the tracers. Tissue sections and calibrated iodine-131 standards were first exposed for 24 hours to x-ray film (Kodak NMB, Rochester, New York) to record iodine-131 tissue radioactivity. Two months later (i.e., after complete decay of the iodine-131), the same sections and calibrated carbon-14 plastic standards were exposed for 2 weeks to the same film for autoradiographic recording of total tissue carbon-14 radioactivity. The absence of cross-contamination between iodine-131 and carbon-14 autoradiograms was verified by reexposure of the same sections for 24 hours and confirmed by the absence of blackening of the film. Finally, tissue sections were washed incubated in 10% trichloroacetic acid (TCA) to remove acid-soluble carbon-14 radioactivity and exposed again for 2 weeks to Kodak NMB film to determine protein-incorporated carbon-14 radioactivity. Optical densities of the autoradiograms were measured with a rotating densitometer (Scandic 3, Joyce Loebl, Gateshead, UK) connected to an image processor (ID 2000, DeAnza Systems, Fremont, California) and a laboratory computer (PDP 11/24, Digital Equipment Corp., Maynard, California). The tissue isotope radioactivity was quantified from the optical density/radioactivity relation as determined with isotope plastic standards.

Regional cerebral blood flow was calculated according to Reivich et al.\(^{18}\) as

$$C_i^* (T) = F \int_0^T C_i^* (t) e^{-\frac{t}{\lambda}} dt$$

where $C_i^* (T)$ is the local iodine-131 tissue radioactivity determined by means of quantitative autoradiography, $C_i^* (t)$ is the arterial blood activity, $F$ is the blood flow per unit tissue weight per minute, and $\lambda=0.8$ is the tissue–blood partition coefficient of IAP.\(^{19}\) Regional blood flow was determined in the cerebral cortex, caudate/putamen, hippocampus, and thalamus. Local blood flow was also measured in various areas and correlated with amino acid incorporation into proteins as determined by carbon-14 autoradiography of the same tissue sections.

Amino acid incorporation into proteins was measured by quantitative autoradiography and by conventional biochemical methods in tissue samples taken from the frontal and parietal cortices of both hemispheres.

Protein synthesis was evaluated autoradiographically by determining the ratio of TCA-insoluble to total radioactivity (fractional protein radioactivity) by digitizing carbon-14 autoradiograms obtained from the same tissue sections before and after wash-incubation with TCA and by dividing the corresponding radioactivities on a pixel-by-pixel basis using the image processing system.
The tissue samples taken for biochemical analysis were weighed and sonified in distilled water. The TCA-insoluble materials were precipitated by addition of an equal volume of 10% TCA to the homogenate. Pellets were resolubilized in 2% Na₂CO₃ and carbon-14 radioactivity was determined in a liquid scintillation counter (LS 7000, Beckman). Aliquots of the samples were used to determine protein content according to Lowry et al. 20

The TCA-soluble supernatant was divided into two parts. One part was taken for determination of total TCA-soluble carbon-14 radioactivity; the other was used for measurement of the specific radioactivity of free leucine using high-performance liquid chromatography (HPLC, Waters Chromatography, Eschborn, FRG). For this purpose, samples were extracted with ether to remove TCA and subsequently evaporated to dryness. Precolumn derivatization was carried out with dansyl chloride according to Badoud and Pratz. 21 Amino acids were analyzed on a reverse-phase column (Brownlee RP-300, 250×4 mm, Kontron Instruments, Eching, FRG) with a linear gradient of 10% to 85% 2-isopropanol in acetate buffer (pH 2.6). The leucine fraction was collected and its carbon-14 radioactivity was measured in the liquid scintillation counter.

Plasma samples taken during incorporation of [14C]leucine were deproteinized with 5% TCA and processed in the same way as tissue samples for obtaining time-integrated specific activity of free leucine in arterial plasma.

The nonparametric Wilcoxon-Mann-Whitney U test was used to test differences between amino acid incorporation in the left and right hemispheres. The relation between cerebral blood flow and amino acid incorporation into brain proteins was analyzed by submitting grouped blood flow values and corresponding fractional protein radioactivities to the nonparametric Kruskal-Wallis test, followed by multiple comparisons of independent samples according to Nemenyi (see Sachs22).

Results

The IAP autoradiograms of control gerbils did not reveal any abnormalities; blood flow in gray matter structures was 180–220 ml/100 g/min, and no significant differences between hemispheres were detected. Blood flow in the right (contralateral) hemisphere of ischemic gerbils was moderately reduced to 120–140 ml/100 g/min; in the left (ipsilateral) hemisphere, blood flow sharply declined from the medial to the lateral portions (Figure 1). In the parietal and temporal cortices, blood flow was reduced to <10 ml/100 g/min. Perfusion in the medial parts of the caudate/putamen and the thalamus was more heterogeneous and ranged between 10 and 40 ml/100 g/min (Figure 1).

In control gerbils the integrated specific activity of leucine in blood plasma was 12.68 nCi/nmol. Thirty minutes after application of tracer [14C]-leucine, the specific activity of free leucine in the brain was 0.07±0.03 nCi/nmol (Table 1).

The total brain radioactivity of control gerbils after 30 minutes of incorporation was approximately 76 nCi/g wet wt. Approximately 70% of the radioactivity was incorporated into brain proteins and 10% was associated with free leucine. The remaining 15% was recovered during the first 3 minutes of HPLC analysis, indicating that this fraction consisted of metabolites of low molecular weight.

The integrated plasma radioactivity of ischemic gerbils did not differ from that of controls. In the contralateral hemisphere, the total carbon-14 radioactivity increased slightly but the incorporation pattern was similar to that of controls. In the ipsilateral hemisphere, in contrast, this pattern substantially changed. The fractional radioactivity incorporated into proteins decreased from 70% to <15%, and the radioactivity of free leucine increased from 10% to >60%. The tissue content of free leucine increased by approximately 80%, indicating uncoupling of amino acid transport across the blood–brain

FIGURE 1. Local cerebral blood flow (CBF) and protein synthesis of gerbil brain measured by double-tracer autoradiography. Iodine-131 autoradiograms (top) show decrease of CBF in ipsilateral hemisphere after left common carotid artery occlusion; in center of brain, CBF exhibited some heterogeneity. Carbon-14 autoradiograms without trichloroacetic acid washing (middle) demonstrate film blackening in ipsilateral hemisphere due to high content of free [14C]leucine, which obscured recognition of inhibition of protein synthesis. Bottom: Autoradiograms from same tissue sections after wash-incubation; ischemic and nonischemic regions are now sharply demarcated.
Table 1. ([14C]Leucine Incorporation Into Brain Proteins of Gerbils After Left Common Carotid Artery Occlusion

<table>
<thead>
<tr>
<th></th>
<th>Control gerbils (n=4)</th>
<th>Contralateral hemisphere (n=6)</th>
<th>Ipsilateral hemisphere (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain radioactivity (nCi/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75.96±6.63</td>
<td>90.97±10.40</td>
<td>55.38±6.85*</td>
</tr>
<tr>
<td>Acid-precipitable</td>
<td>52.53±14.46</td>
<td>73.96±9.93</td>
<td>7.11±0.79*</td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrated plasma specific activity (nCi/nmol)</td>
<td>12.68</td>
<td>13.78±3.24</td>
<td></td>
</tr>
<tr>
<td>Brain concentration (nmol/mg)</td>
<td>0.14±0.04</td>
<td>0.12±0.03</td>
<td>0.24±0.09†</td>
</tr>
<tr>
<td>Brain specific activity (nCi/nmol)</td>
<td>0.07±0.03</td>
<td>0.07±0.04</td>
<td>0.14±0.03†</td>
</tr>
</tbody>
</table>

Values are mean±SD. n, number of hemispheres. *p<0.001, 0.005, respectively, different from control by Wilcoxon-Mann-Whitney U test.
values as in the other structures. At blood flows of <40 ml/100 g/min, amino acid incorporation into proteins was very low or zero; at blood flow values of >100 ml/100 g/min, protein synthesis was normal.

Discussion

The use of amino acid incorporation for evaluating brain protein synthesis after vascular occlusion requires some methodologic considerations that should be discussed before proceeding to the interpretation of the results. A kinetic approach for quantification of protein synthetic rate is not possible under ischemic conditions because tracer supply changes with blood flow and because the kinetic constants for the transport of amino acids across the blood-brain barrier and for the aminoacylation of transfer ribonucleic acid (tRNA) may also change. For this reason, we directly measured the distribution of the tracers in the various metabolic compartments in addition to autoradiographically evaluating amino acid incorporation into brain proteins.

In arterial blood, the integrated specific activity of leucine did not vary between control and ischemic gerbils, and corrections for differences in tracer concentrations were not required. In the brain, in contrast, considerable differences existed, the content and specific activity of free leucine being significantly higher in the ipsilateral hemisphere. This finding demonstrates that despite the reduction in blood flow, tracer supply was enhanced and not limited in relation to the utilization of amino acids.

The high free leucine content cannot be related to an enhanced degradation of brain proteins during ischemia because this would induce a dilution of radiolabeled tracer in the precursor pool and, in consequence, a fall in specific activity. The contrary was observed, which suggests that either protein catabolism is inhibited after vascular occlusion or that the transport of amino acids is increased. Inhibition of the catabolism of proteins with short half-lives has previously been observed during ischemia in monkey brain and may also prevail in our situation. An increase in transport is less likely because the total tissue radioactivity (i.e., incorporated and nonincorporated tracer) was slightly lower after vascular occlusion (Table 1). The observed changes in the free leucine pool, in consequence, simply reflect the accumulation of nonincorporated precursor.

This conclusion is of great importance for the interpretation of the observed threshold relation between blood flow and protein synthesis. The beginning decline in amino acid incorporation at blood flows as high as 100 ml/100 g/min cannot be explained by the change in amino acid transport or a decrease in specific activity of the precursor pool but represents a true inhibition of the protein synthesizing machinery. In fact, because of the high specific activity of the precursor, the suppression of protein synthesis was even more severe than apparent from the remaining amino acid incorporation rate. Similarly, the complete suppression of protein synthesis at blood flows of <40 ml/100 g/min cannot be explained by kinetic abnormalities and, therefore, represents the absolute ischemic threshold of protein synthesis.

Our observation of a remarkably high threshold of blood flow for disturbances of protein synthesis...
is in line with a previous study of Jacewicz et al., who measured protein synthesis and blood flow in tissue samples taken from various brain regions after occlusion of the carotid and middle cerebral arteries in rats. Although a detailed threshold analysis was not carried out in that study, the results seem to indicate that mild blood flow reductions, to 50–80 ml/100 g/min, are accompanied by a beginning inhibition of the synthesis of many proteins and that with further reduction, to 40 ml/100 g/min, synthesis of most proteins is suppressed.

The threshold for inhibition of protein synthesis, in consequence, is much higher than that for other physiologic and metabolic functions of the brain. Evoked potentials are suppressed at blood flows of approximately 15 ml/100 g/min, adenosine 5’-triphosphate (ATP) is depleted below 12 ml/100 g/min (W. Paschen, G. Mies, K-A. Hossmann, unpublished observations), and cell membranes depolarize at blood flows of <10 ml/100 g/min. The comparison of these thresholds clearly demonstrates that suppression of protein synthesis is not caused by either energy failure or major disturbances of intracellular ion concentration but must be due to minor alterations of cell homeostasis. Such a change could be a slight shift in the intracellular guanosine triphosphate:guanosine diphosphate ratio, which has a major effect on the activity of the polypeptide chain initiation factor eIF2. Another factor could be a change in intracellular pH, which declines before ATP decreases and which has been shown to reduce the rate of protein synthesis in vitro.

Another consideration is the down-regulation of protein synthesis at the transcriptional level. In fact, selective gene expression of new polypeptides including a variety of heat-shock proteins has been observed during and after ischemia. However, during focal ischemia in gerbils no gross abnormalities of RNA synthesis are present in regions with suppressed protein synthesis. The pathogenetic role of transcriptional abnormalities, in consequence, remains to be clarified.

An interesting conclusion from our present investigation is that suppression of protein synthesis is markedly reduced in the penumbra of an infarct. The penumbra has been defined as an area with a blood flow between the thresholds for energy failure and electrical failure, which at normal body temperature occur at approximately 10 and 15 ml/100 g/min, respectively. In this range protein synthesis is completely suppressed, which implies that the neurons are not able to survive for extended periods unless blood flow is substantially improved or unless the threshold for the inhibition of protein synthesis decreases over time. The penumbra neurons, therefore, may suffer a pathology similar to those in the selectively vulnerable CA1 sector of the hippocampus after transient ischemia. In the hippocampus, protein synthesis is irreversibly suppressed despite normal energy metabolism and results in the phenomenon of delayed neuronal death after 2–3 days. If the mechanisms of inhibition of protein synthesis in the penumbra of an ischemic focus and in the hippocampus after transient ischemia are similar, similar therapeutic approaches may also be useful for the prevention of delayed neuronal death. This could explain why certain drugs such as calcium or glutamate antagonists reduce not only the number of damaged neurons in the hippocampus after transient global ischemia but also the size of an infarct after permanent focal ischemia.

In conclusion, our study demonstrates that the blood flow threshold for maintenance of cerebral protein synthesis is much higher than for most other neuronal functions. In the ischemic penumbra, protein synthesis is completely suppressed and, in consequence, must lead to neuronal destruction unless a reversal of the suppression occurs over time. Treatment of cerebral infarcts, therefore, should consider not only the maintenance or restoration of energy state and ion homeostasis but also the more widespread alterations of protein synthesis in the periphery of the ischemic territory.

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**KEY WORDS** • autoradiography • cerebral blood flow • gerbils
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