Effect of 5-Minute Ischemia on Regional pH and Energy State of the Gerbil Brain: Relation to Selective Vulnerability of the Hippocampus

K. Munekata and K.-A. Hossmann

Adult male gerbils were submitted to 5-minute cerebral ischemia by bilateral carotid artery occlusion. At the end of ischemia and at various recirculation times ranging from 15 to 120 minutes, brains were frozen in situ and the regional distribution of ATP, glucose, and tissue pH was studied on coronal cryostat sections by bioluminescent and fluoroscopic techniques. During ischemia ATP was completely depleted, glucose decreased to <10% of control, and regional tissue pH decreased from 7.04–7.09 to about 6.0. After the beginning of recirculation tissue pH and the regional content of metabolites exhibited a triphasic course. After 15 minutes pH returned to or even above normal, and ATP- and glucose-induced bioluminescence normalized. However, there was a secondary deterioration of both tissue acidosis and the metabolic state after 30 minutes. After longer recirculation times changes again improved and returned to normal within 2 hours. These changes were similar in all brain regions with the exception of the CA1 subfield of the hippocampus, where the transient normalization of tissue pH was absent after 15 minutes of recirculation. This finding is in line with the previously observed microcirculatory insufficiency of this area and demonstrates that the CA1 sector of the hippocampus suffers more pronounced postischemic acidosis than other less vulnerable regions of the brain. (Stroke 1987;18:412–417)

During the past years, the bilateral carotid artery occlusion model for production of global cerebral ischemia of gerbils has been widely used to study the phenomenon of selective vulnerability in the CA1 subfield of the hippocampus. A brief ischemic period of only 5 minutes results in persisting disturbances of protein biosynthesis of CA1 pyramidal cells, and, after a "maturation" interval of about 2 days, in delayed death of these cells. Hemodynamic and metabolic studies revealed that neither blood flow nor energy metabolism is critically reduced during maturation, indicating that the phenomenon of selective vulnerability cannot be explained by an energy crisis induced by postischemic recirculation disturbances. Instead, specific molecular mechanisms have been made responsible for the phenomenon. Data collected from various experimental models and different animal species suggest that neuronal hyperexcitability, the release of excitotoxins, intracellular calcium overload, and alterations of gene expression of protein synthesis may be involved. The main argument in favor of such a mechanism is the fact that the surgical disconnection of excitatory input, the application of certain antagonists of excitotoxins, or the application of calcium antagonists alleviates the pathological process. It should be remembered, however, that the same procedures may also increase regional blood flow or reduce the metabolic rate and hence the substrate requirements of the endangered territory. In fact, reduction of metabolic rate by barbiturate anesthesia reduces the injury. A hemodynamic contribution to the injury is also indicated by the fact that the angioarchitecture of the CA1 sector—and to a lesser degree also of the CA3 subfield—is significantly less dense than that of the more resistant cerebral cortex and that there is less recruitment of capillaries in the vulnerable regions during postischemic reperfusion.

These observations led us to reconsider the possibility that the microcirculation of the hippocampus is unable to maintain an adequate exchange of substrates between blood and tissue during the critical period of postischemic recovery. To overcome the technical difficulty of tissue sampling from such small regions as the CA1 pyramidal cell layer, we used bioluminescent and fluoroscopic techniques for imaging alterations of substrates and tissue pH on intact brain sections. The following observations demonstrate that there is, in fact, a critical period of postischemic reperfusion during which the energy state and the acid–base homeostasis are disturbed and that these alterations are more pronounced in the vulnerable CA1 sector than in the more resistant regions of the brain.

Materials and Methods

Thirty adult male gerbils of about 70 g body weight were used. Animals were bred in our laboratory and had free access to water and standard rodent diet. Five gerbils were controls, and 25 gerbils were used for production of transient ischemia of the brain. Animals were anesthetized with 2% halothane, the body temperature kept constant at 37° C using a feedback-controlled heating system. In control animals anesthesia was maintained for 30 minutes, and their brains were
frozen in situ by submerging the heads into liquid nitrogen. In experimental animals both carotid arteries were occluded for 5 minutes with small aneurysm clips. After release of the clips, the skin incision was sutured and anesthesia was discontinued. After predetermined recirculation times of 15, 30, 60, and 120 minutes, the gerbils were reanesthetized with 2% halothane, and their brains were frozen in situ as in controls. After removal, the brains were cut in a cryostat, and 20-μm coronal sections were processed for estimating the regional distribution of pH, ATP, and glucose according to previously published techniques.\textsuperscript{16-18}

In short, for measuring tissue pH, cryostat sections were placed on electrophoresis paper soaked with the fluorescent pH indicator umbelliferone and brought to 0°C in an ice bath. After melting, 450 nm fluorescence was recorded photographically after excitation with 370 and 340 nm, and the ratio of the densities of both images was related to tissue pH, using a nomogram obtained with appropriate standards.

ATP and glucose were measured by covering cryostat sections with 60-μm sections of a frozen reaction mix containing all enzymes, coenzymes, and substrates necessary for evoking substrate-specific bioluminescence. The sandwich was placed in the dark on photographic film and warmed to room temperature, thus allowing the reaction mix to diffuse into the tissue section. To allow semiquantitative evaluation of bioluminescent images, cryostat sections of all brains were processed at the same time using the same reaction mix, the same film batch, and the same chemicals for development of films. Optical densities of films were scanned with a rotating densitometer (Scandig 3, Joyce-Loebl) connected to a computer-controlled image processing system (DeAnza ID 1222 and PDP 11-24, Digital Equipment).

Statistical differences of grouped data were analyzed using the nonparametric Wilcoxon \(U\) test.

**Results**

At the end of the 5-minute bilateral carotid artery occlusion period, most areas of the forebrain exhibited severe metabolic alterations (Figures 1–3). Tissue pH decreased from 7.04–7.09 to about 6.0, ATP-induced bioluminescence was zero, and glucose-induced bioluminescence was <10% of control (Table 1). There were no major differences between the cerebral cortex, hippocampus, and thalamus, indicating that the biochemical impact was comparable in vulnerable and nonvulnerable regions. Only in the medial parts of the basal ganglia were metabolic changes less pronounced, which is consistent with the previous documentation of preserved blood flow in these regions. Measurements from these areas, therefore, were not included in the table.

During recirculation, tissue pH and the content of metabolites exhibited a triphasic course (Figures 4–6). After 15 minutes, pH returned to or even above normal, and ATP- and glucose-induced bioluminescence normalized. However, there was a secondary deterioration of both tissue acidosis and the metabolic state after 30 minutes. Later, values again improved and returned to normal within 2 hours. These changes were similar in all areas except the CA1 sector of the hippocampus where the transient normalization of tissue pH was absent after 15 minutes of recirculation. This finding is in line with the previously observed microcirculatory insufficiency of this area\textsuperscript{15} and demonstrates that the CA1 sector of the hippocampus suffers more pronounced acidosis than other less vulnerable regions of the brain.

**Discussion**

Before we try to interpret the present findings, a few methodologic remarks should be made. The bioluminescent images of ATP content of gerbil brain before and after 5-minute bilateral carotid artery occlusion (high substrate concentration is indicated by bright bioluminescence). A, control; B, 5-minute ischemia; C, 15-minute recirculation; D, 2-hour recirculation. Note depletion of ATP during ischemia in all regions except the central parts and homogeneous recovery upon recirculation. There is no evidence of regional differences between the selectively vulnerable hippocampus and other parts of the brain.

**Discussion**

Before we try to interpret the present findings, a few methodologic remarks should be made. The bioluminescent images of glucose content of gerbil brain before and after 5-minute bilateral carotid artery occlusion (high substrate concentration is indicated by bright bioluminescence). A, control; B, 5-minute ischemia; C, 15-minute recirculation; D, 2-hour recirculation. Changes of glucose content resemble those of ATP (see Figure 1). Note absence of regional differences between the hippocampus and other parts of the brain.
Fig. 3. Images of gerbil brain tissue pH before and after 5-minute bilateral carotid artery occlusion (low tissue pH is indicated by bright signal). A, control; B, 5-minute ischemia; C, 15-minute recirculation; D, 2-hour recirculation. Note homogeneous decrease of tissue pH during ischemia in all areas but delayed normalization of pH in the hippocampus and thalamus, indicated by the difference in signal intensity after 15-minute recirculation.

Bioluminescent techniques used for regional measurement of ATP and glucose are semiquantitative, and they are primarily intended for imaging regional differences rather than absolute changes of substrate levels. However, the stoichiometry of the enzymatic reaction exhibits a direct relation between substrate concentration and light emittance, which in turn correlates linearly with the optical density of the exposed photographic film. By using appropriate standards or measuring the substrate content of circumscribed regions of the tissue block with conventional fluorometric methods, it is possible to calibrate the bioluminescent images for quantitative evaluation. In the present study such a calibration was not carried out, but the cryostat sections of all animals were processed at the same time with the same enzyme block, using the same film batch and the same chemicals for film development. It is, therefore, possible to express the metabolic changes in percent of control material. The pH images rely on a different principle; they are quantitative and the numbers presented are absolute values. However, this technique also has methodologic restrictions. pH-induced fluorescence is quenched by hemoglobin, and large increases of blood volume result in an erroneously low pH. During ischemia blood volume decreases, and after ischemia it transiently rises as a consequence of postischemic hyperemia. The observed pH changes are in the opposite direction, and the reported values, therefore, are slight underestimates of the actual variations. On the other hand, ischemia-induced changes of the ratio of extracellular to intracellular pH tend to slightly accentuate the alterations of tissue pH. Under physiologic conditions, the extracellular compartment is about 20% of total brain volume, and extracellular pH is about 0.3 units higher than intracellular pH. Total tissue pH, in consequence, is about 0.06 units higher than intracellular pH. At the end of ischemia the extracellular compartment is reduced to about 10% of brain volume; assuming a constant difference between extra- and intracellular pH, total tissue pH would now be only 0.03 units higher than intracellular pH. These variations, however, are small compared with the observed pH shifts of about 1.0 unit.

With these methodologic considerations in mind, the results obtained are interpreted as follows: The changes observed at the end of the ischemic period are

Table 1. Regional Evaluation of ATP, Glucose, and Tissue pH Before and at Various Recirculation Times After 5-Minute Cerebral Ischemia

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>Ischemia (n = 5)</th>
<th>15 minutes (n = 5)</th>
<th>30 minutes (n = 5)</th>
<th>1 hour (n = 5)</th>
<th>2 hours (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cortex</td>
<td>0.33 ± 0.12</td>
<td>0.00 ± 0.00*</td>
<td>0.38 ± 0.27</td>
<td>0.13 ± 0.08†</td>
<td>0.22 ± 0.16</td>
<td>0.39 ± 0.16</td>
</tr>
<tr>
<td>CA1 sector</td>
<td>0.39 ± 0.11</td>
<td>0.00 ± 0.00*</td>
<td>0.41 ± 0.30</td>
<td>0.21 ± 0.10</td>
<td>0.29 ± 0.21</td>
<td>0.41 ± 0.14</td>
</tr>
<tr>
<td>CA3 sector</td>
<td>0.47 ± 0.11</td>
<td>0.00 ± 0.00*</td>
<td>0.50 ± 0.18</td>
<td>0.34 ± 0.07</td>
<td>0.37 ± 0.17</td>
<td>0.48 ± 0.14</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.51 ± 0.14</td>
<td>0.00 ± 0.01*</td>
<td>0.54 ± 0.20</td>
<td>0.26 ± 0.12</td>
<td>0.39 ± 0.18</td>
<td>0.62 ± 0.18</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>0.49 ± 0.05</td>
<td>0.03 ± 0.02*</td>
<td>0.65 ± 0.10</td>
<td>0.54 ± 0.16</td>
<td>0.63 ± 0.06</td>
<td>0.53 ± 0.05</td>
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<tr>
<td>CA1 sector</td>
<td>0.47 ± 0.04</td>
<td>0.04 ± 0.03*</td>
<td>0.60 ± 0.17</td>
<td>0.40 ± 0.16</td>
<td>0.57 ± 0.21</td>
<td>0.46 ± 0.06</td>
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<tr>
<td>CA3 sector</td>
<td>0.45 ± 0.05</td>
<td>0.03 ± 0.02*</td>
<td>0.63 ± 0.13</td>
<td>0.45 ± 0.17</td>
<td>0.65 ± 0.22</td>
<td>0.49 ± 0.07</td>
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<tr>
<td>Thalamus</td>
<td>0.57 ± 0.05</td>
<td>0.14 ± 0.10*</td>
<td>0.80 ± 0.09</td>
<td>0.59 ± 0.18</td>
<td>0.72 ± 0.17</td>
<td>0.53 ± 0.10</td>
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<td>Tissue pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>7.04 ± 0.09</td>
<td>5.93 ± 0.15*</td>
<td>7.13 ± 0.16</td>
<td>6.87 ± 0.12†</td>
<td>7.11 ± 0.14</td>
<td>7.12 ± 0.11</td>
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<td>CA1 sector</td>
<td>7.07 ± 0.04</td>
<td>5.97 ± 0.13*</td>
<td>6.82 ± 0.20*</td>
<td>6.84 ± 0.12</td>
<td>6.94 ± 0.15</td>
<td>7.14 ± 0.09</td>
</tr>
<tr>
<td>CA3 sector</td>
<td>7.08 ± 0.04</td>
<td>5.99 ± 0.15*</td>
<td>7.01 ± 0.24</td>
<td>6.94 ± 0.12</td>
<td>7.07 ± 0.10</td>
<td>7.09 ± 0.07</td>
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<tr>
<td>Thalamus</td>
<td>7.09 ± 0.05</td>
<td>6.01 ± 0.18*</td>
<td>6.95 ± 0.25</td>
<td>6.79 ± 0.09*</td>
<td>7.06 ± 0.14</td>
<td>7.10 ± 0.04</td>
</tr>
</tbody>
</table>

Changes of ATP and glucose are expressed as optical densities of bioluminescent images (means ± SD).

*Different from control at p < 0.05, **p < 0.10, respectively (Wilcoxon U test).
FIGURE 4. Quantitative evaluation of ATP-induced tissue bioluminescence in various regions of gerbil brain before and after 5-minute bilateral carotid artery occlusion. Bioluminescence is expressed as optical density of exposed photographic film (see "Materials and Methods"). Note initial normalization of ATP in all regions of the brain followed by secondary transient decline between 30 and 60 minutes of recirculation. Values are means ± SEM.

FIGURE 5. Quantitative evaluation of glucose-induced tissue bioluminescence in various regions of gerbil brain before and after 5-minute bilateral carotid artery occlusion. Similar time course as ATP (see Figure 4). Note absence of differences between the hippocampus and other parts of the brain. Values are means ± SEM.

FIGURE 6. Quantitative evaluation of tissue pH in various regions of gerbil brain before and after 5-minute bilateral carotid artery occlusion. During ischemia pH decreases to similar levels in all regions, but it is significantly lower in the CA1 sector of the hippocampus than in other areas of brain during the early recirculation period. This difference disappears after longer recirculation times. Values are means ± SEM.

in agreement with earlier results obtained by tissue sampling techniques21,22 and confirm that 5-minute bilateral carotid artery occlusion in gerbils causes a severe ischemic injury that affects almost the entire volume of the forebrain.

The decrease of tissue pH during ischemia to between 5.93 and 6.0 ranks in the lower range of previously reported values; Ljunggren et al23 calculated a decrease of intracellular pH at the end of 5-minute intracranial hypertension in rats to between 6.0 and 6.8 depending on plasma glucose levels, and Siemkowicz and Hansen24 measured a decrease of extracellular pH during 10-minute complete ischemia in rats to between 6.13 and 6.55. Measurements in gerbils, to our knowledge, have previously been reported only by Thulborn et al.25 Using nuclear magnetic resonance (NMR) spectroscopy, these authors measured a fall of pH after carotid ligation to only 6.61, but their control value of 7.2 was distinctly higher than that observed in most other laboratories. We, therefore, conclude that the actual decrease of tissue pH during 5-minute ischemia of the gerbil brain ranges to somewhere between 6.0 and 6.5.

During postischemic recirculation, energy state and pH normalized in the cerebral cortex within 15 minutes, which is also in line with previous observations24-28 The new findings of the present study are 1) the persisting relative acidosis in the CA1 sector of the hippocampus during the early recirculation period, and 2) the secondary global deterioration of the energy
state and acid–base balance in both the resistant and vulnerable regions of the brain.

The persisting acidosis in the CA1 sector contrasts with the rapid normalization of ATP and glucose, which indicate that blood flow and metabolic activity are resumed in this region and that the supply of nutrients covers the energy demands of the tissue. However, the tissue content of ATP does not provide information about the metabolic rate or the pathways used for generation of energy-rich substrates. Recently, evidence has been provided by NMR spectroscopy that lactate may transiently increase further during early postischemic recirculation, indicating that anaerobic glycolysis persists despite reoxygenation of the tissue. It is likely that this effect is enhanced by inhomogeneous blood reperfusion of the tissue. In fact, morphometric studies have revealed that the number of perfused capillaries is significantly lower in the CA1 sector of the hippocampus than in the cerebral cortex or the nonvulnerable regions of the hippocampus. The persisting acidosis of the hippocampus, in consequence, could be the result of ongoing ischemic glycolysis, which is manifested selectively in the CA1 sector because of the particular hemodynamic situation in this region.

The global secondary deterioration of energy state and acid–base balance after 30–60 minutes of recirculation in both the resistant and vulnerable regions is probably related to a different phenomenon. The metabolic impairment correlates with the previously documented postischemic hypoperfusion.

There is evidence from separate experiments that oxidative glucose utilization recovers during this state, but the high metabolic demands of the now electrically active brain and the partial uncoupling of oxidative phosphorylation may cause a misrelation between oxygen demands and availability, resulting in relative tissue hypoxia and secondary stimulation of "anaerobic" glycolysis. The degree of secondary hypoxia is presumably minimal because the disturbance is reversed within 2 hours. In fact, previous experiments of similar or even longer durations of ischemia carried out in awake or barbiturate-anesthetized gerbils led to less severe secondary deterioration of energy state, probably because of a slightly less severe degree of postischemic hypoperfusion and/or less severe uncoupling of oxidative phosphorylation.

The importance of the observed alterations of tissue pH and energy state for the development of selective vulnerability is difficult to estimate. The only difference between vulnerable and resistant areas observed in the present study was relative acidosis in the CA1 sector during the early recirculation period. However, the absolute pH measured in this region was not much lower than in the resistant areas during subsequent postischemic hypoperfusion phase. It is, therefore, unlikely that the observed degree of tissue acidosis per se is responsible for delayed neuronal cell death. However, since pH was not continuously measured we may have missed the peak value of postischemic acidosis. Moreover, delayed cell death is also associated with other metabolic disturbances, such as persisting inhibition of protein synthesis. It is conceivable that regional variations of pH during the early recirculation phase may be of importance for the development of this lesion, but without more precise information about the molecular mechanisms involved the postulation of a causal relationship would be purely speculative. The delayed reversal of tissue pH in the selectively vulnerable CA1 sector of the hippocampus, therefore, may be an epiphenomenon of other ongoing pathologic processes.

Acknowledgments

The authors thank Mr. Göldner for preparation of the figures, and Mrs. Hahmann and Mrs. Göldner for careful preparation of the manuscript. The facilities for preparation of the bioluminescent images were kindly provided by Dr. W. Paschen.

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**Key Words** • pH • energy metabolism • ischemia • gerbil • selective vulnerability
Effect of 5-minute ischemia on regional pH and energy state of the gerbil brain: relation to selective vulnerability of the hippocampus.

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Stroke. 1987;18:412-417
doi: 10.1161/01.STR.18.2.412

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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