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References


Cerebral Ischemia in Gerbils: Polyribosomal Function During Progression and Recovery

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SUMMARY Cerebral ischemia was produced by clipping the right common carotid artery in Mongolian gerbils. Polyribosomal function in cerebral ischemia during progression and recovery was studied by investiga-
tion of morphology (electronmicroscopy), physical property (size distribution profiles) and biochemical proper-
ty (polypeptide synthesis). Polyribosomes and their function were relatively well preserved during progression of ischemia. The most striking finding was an extensive disaggregation of polyribosomes and suppression of polypeptide synthesis immediately after re-establishment of cerebral circulation. These phenomena occurred not only with irreversible ischemia at 3 h but also with reversible ischemia at 30 min. In the latter, disaggrega-
tion of polyribosomes gradually recovered, but no tendency for recovery was observed after an ischemic period of 3 h. The disaggregation and delay in reaggregation of ribosomes after re-establishment of cerebral circulation may be a significant factor in the irreversibility of cerebral ischemia. The observed deterioration of cellular function during the recovery process may have an important implication not only for medical manage-
ment of stroke but also for surgical recirculation during acute stroke.

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leading to the observed dysfunction is not clear. In the present study, we further evaluated the polyribosomal function during progression and recovery from cerebral ischemia after occlusion of the right common carotid artery in gerbils. The physical properties of polyribosomes were investigated by the size distribution profiles and biochemical property by polypeptide synthesis. To confirm the alteration seen in the size distribution profiles, brain tissue was further investigated by electronmicroscopy. The most striking finding was the loss of polyribosomal integrity and function immediately after re-establishment of cerebral circulation with various subsequent recovery processes, which were reflected in all 3 parameters investigated here. (The present study has been reported in abstract. 10)

Materials and Methods

Preparation of Pathological Condition

Each gerbil (60–70 gm) was anesthetized by ether inhalation. The right common carotid artery was exposed through a midline incision in the neck, freed from connective tissue and the vagus nerve, and clipped with a miniature Mayfield aneurysmal clip. The wound was closed with a surgical skin clip. Post-operatively each animal was observed for clinical signs such as torsion of the neck, circling, rolling and drowsiness for up to 3 h. The animals designed for the study of the recovery process were reanesthetized with ether, and the clip removed. Resumption of blood flow in the previously occluded artery was ascertained visually, and the incision closed. The gerbils were further observed for 15 min to 3 h.

Brains were removed by decapitation and quickly cooled in crushed ice. The right cerebral hemisphere served as the experimental sample and the left one as the control sample. The left cerebral hemisphere of an asymptomatic animal was also taken as a control. The subsequent procedures were carried out at 4°C. The experimental and control samples were processed simultaneously. The animals with and without recovery period were processed together except for those with ischemia for 30 min and recovery for 15 min.

Preparation of Polyribosomes, Microsomes and pH 5 Factor

The procedure for preparation of polyribosomes is based on the method of Weiss et al. 14 as described previously 12 with some modification. Cerebral hemispheres were homogenized with 0.25M sucrose (ribonuclease-free) in TKMM buffer and centrifuged in a Beckman SW 56 rotor at 105,000 g (average) for 90 min. The gradient was recovered from the top and recorded at 260 nm in a Beckman DB-GT recording spectrophotometer through a continuous flow cell. 12 The peak area of monomer was determined by using bacteriophage MS2 which has a modal S value of 80. In some instances, the outflow from the flow cell was collected in fractions and the sedimentation coefficient of the second major peak designated as dimer was calculated by the method of McEwen. 16 The relative size of specific peaks (polysomes, dimer, and monomer) were determined by weighing each peak and expressed as percent of total ribosomes. Ribosomes beyond dimer were taken as polysomes.

Size Distribution Profiles of Polyribosomes

The suspension equivalent to 3.0 A 260 units was layered onto 3.8 ml linear sucrose gradient (10–60%) in TKMM buffer and centrifuged in a Beckman Type Ti70 rotor. The resulting polyribosomal pellet was resuspended in TKMM buffer and centrifuged at 222,000 g for 200 min in a Beckman Type Ti70 rotor. The resulting polyribosomal pellet was resuspended in TKMM buffer. The content of polyribosomes from each suspension was determined by UV absorbance at 260 nm (A 260 unit), and the concentration of polyribosomes in the control and experimental sample were made equal. For preparation of microsomes, cerebral hemispheres were homogenized with 0.25M sucrose containing Tris-HCl, 10 mM at pH 7.4; KCl, 10 mM; MgCl2, 15 mM. Microsomes and supernatant were separated by centrifugation of postmitochondrial supernatant at 150,000 g for 90 min. 18 After suspension in the same sucrose solution, protein content of microsomes was determined according to Lowry et al. 14. The pH 5 factor was prepared from normal brain by centrifugation of the postmitochondrial supernatant along with polyribosomes or microsomes and by precipitation of the resulting supernatant at pH 5 as described previously. 18 The protein concentration was determined according to Lowry et al. 14 for microsomes, and as A 260 unit by absorbance at 280 nm for polyribosomes. 12

Polypeptide Synthesis with Cell-Free Systems

Polypeptide synthesis was carried out in the incubation medium consisting of 50 mM Tris-HCl buffer, pH 7.4, 50 mM KCl, 10 mM MgCl2, 10 mM 2-mercaptoethanol, 2 mM ATP, 0.2 mM GTP, 10 mM phosphoenolpyruvate, 16 unit pyruvate kinase, 0.1 mM each 19 L-amino acid mixture and 5 µCi of L-[4,5-3H] leucine (specific activity 46–59 Ci/m mole, Amersham, Arlington Heights, IL), modified from Johnson and Belytschko 18 as described previously. 12 For polypeptide synthesis with microsomes, 0.3 mg of microsome protein and 0.5 mg of pH 5 factor were added. For polyribosomes, 0.4 A 260 units of polyribosome and 2.0 A 260 units of pH 5 factor were added. All samples were run in triplicate. Incubation was carried out at 35°C for polyribosomes and 25°C for
microsomes for 20 min and the reaction was terminated by addition of 0.5 ml of cold 10% trichloroacetic acid (TCA) and 3 ml of cold 5% TCA, and the resulting precipitate was washed as described previously. The dried sample was solubilized with Soluene-350 (Packard Instrument Co., Downers Grove, IL) and the radioactivity was counted in toluene-based scintillation fluid in a liquid scintillation spectrometer with 45% efficiency. The result was expressed as disintegration per min (dpm)/unit of polyribosomes or microsomes.

**Tissue Preparation for Electron Microscopy**

Brain for electronmicroscopic examination was quickly removed after decapitation and placed on a cooling plate. A coronal section 1 mm thick was taken from the parietal area of the right cerebral hemisphere at approximately the middle point between the frontal and occipital pole, and a small tissue block of approximately 1 mm³ was further dissected out promptly. Each tissue block was immediately placed in ice cold 3% glutaraldehyde in 100 mM phosphate buffer (pH 7.4) for 60 min, rinsed with the same buffer and further fixed for 60 min in 1% osmium tetroxide made in the same buffer. The tissue block was dehydrated in alcohol and embedded in the Spurr embedding medium. An ultrathin section for electronmicroscopic examination was prepared with an LKB ultramicrotome, stained with uranyl acetate and examined with a Phillips 400 transmission electronmicroscope.

**Results**

Animals recovered from anesthesia within 5 min. All symptomatic animals taken for this study had typical signs of cerebral ischemia within 30 min. Seizures were frequent beyond two h of ischemia. During the recovery period after ischemia for 30 min, animals were still slow at 15 min but were nearly normal at 3 h. Animals remained quite symptomatic at 3 h, if the ischemic period was 3 h. The condition of animals rendered ischemic for one h was between the above 2 conditions.

The size distribution profiles of polyribosomes from normal brain is shown in fig. 1 (left). The first peak identified as monomer (M) co-migrated with bacteriophage MS2 which has a modal $S$ value of 80 (fig. 1, left column, bottom). The second peak designated as dimer (D) has a modal $S$ value of 140 according to the calculation formula of McEwen. There was little difference in the size distribution profiles after extended centrifugation of postmitochondrial supernatant for 16 h or when the sample was homogenized in the presence of bentonite (1 mg/ml), a known inhibitor of ribonuclease. An addition of heparin (0.5 mg/ml), another inhibitor of ribonuclease, resulted in partial degradation of polyribosomes to ribosomal subunits.

The results from ischemic samples are shown in the center and right column of fig. 1. The only abnormal finding after an ischemic period of 30 min was a slight increase in the dimer peak (fig. 1, center column, top). After ischemia for 3 h, there was a significant increase of the monomer-dimer complex with concomitant decrease of polysomes (fig. 1, right column, top). The relative size of polysomes was reduced from the normal value of 84% to 61% on quantitative measurement. Soon after re-establishment of cerebral circulation, a very dramatic disaggregation of polysomes and sharp increase in the monomer-dimer complex occurred not only after ischemic period for 3 h (fig. 1, right column, middle) but also after 30 min (fig. 1, center column, middle). Quantitatively the size of polysomes was 36% after an ischemic period of 3 h. Even though there was a significant disaggregation of polysomes immediately after re-establishment of cerebral circulation, the profile returned to near normal after a recovery period of 3 h if the ischemic period was only 30 min (fig. 1, center column, bottom).

**Figure 1.** The size distribution profiles of polyribosomes from normal right cerebral hemispheres (left column), right cerebral hemispheres after ischemia for 30 min (center column) and right cerebral hemispheres after ischemia for 3 h (right column). For normal brain, the profile on the top was made without a marker, the middle one in the presence of 80S marker and the bottom one, the marker only. For ischemic brains, the profiles on the top were without any recovery period, the middle ones 15 min after re-establishment of circulation and the bottom one 3 h after re-establishment of circulation. $OD_{260}$ units shown in ordinate are arbitrary. $M$ and $D$ indicate the monomer and dimer, respectively, and $P$ indicates the polysome peak.
There was no tendency for recovery if the ischemic period was 3 h (fig. 1, right column, bottom). The profiles for an ischemic period of one h were intermediary. The presence of bentonite (1 mg/ml) in the homogenization medium did not modify the observed alterations of polyribosomal profiles. The size distribution profiles from the left cerebral hemispheres of symptomatic animals showed a very slight increase of the dimer peak after an ischemic period of 3 h.

Because of a dramatic disintegration of polyribosomes soon after re-establishment of cerebral circulation, polypeptide synthesis was evaluated with isolated microsomes and polyribosomes with special attention to that particular period. Due to difficulty in producing a sufficient number of symptomatic animals after ischemia for 30 min, enough to run assays for those with and without recovery period simultaneously, an ischemic period of one h was chosen for evaluation of polypeptide synthesis. With the present assay systems, leucine incorporation was approximately 170 dpm/µg protein or 1.5 pmole leucine incorporation/mg protein for normal microsomes and approximately 45,000 dpm/Am unit or 0.4 pmole leucine incorporation/Am unit of normal polyribosomes. The effect of ischemia on polypeptide synthesis is shown in the table. With the microsomal fraction, the suppression of leucine incorporation was over 30% after an ischemic period of one h, which reached 50% after a recovery period of 15 min. After a recovery period of 3 h, a tendency for recovery of various degrees was observed. With the polyribosomal fraction, the suppression of leucine incorporation was significant only after re-establishment of cerebral circulation. There was no significant difference between the left cerebral hemispheres of symptomatic and asymptomatic animals during the ischemic or recovery period investigated here.

To further determine if the observed alterations of physical and biochemical properties really occurred in vivo, brain tissue was examined electronmicroscopically with special attention to ribosomes. After an ischemic period of one h, cortical neurons showed significant swelling of cisterns of endoplasmic reticulum and mitochondria but lysosomes appeared intact. The extent of disaggregation of polyribosomes was difficult to evaluate quantitatively but both aggregated and disaggregated forms appeared to exist. However, polyribosomes became almost completely dispersed after a recovery period for 15 min (fig. 2) even though swelling of endoplasmic reticulum receded and mitochondria appeared unchanged in comparison to those prior to re-establishment of cerebral circulation. It is of interest to note the more obvious swelling of astrocytic processes soon after re-establishment of cerebral circulation.

**Discussion**

Disaggregation of polyribosomes has been observed during the recovery period by Kleihues and Hossmann after prolonged complete occlusion of major arteries in cats and by Cooper et al. following interruption of cerebral circulation by intracranial hypertension in rats. Electronmicroscopically, Hartmann and Becker noted disaggregation of polyribosomes during the recovery stage following temporary occlusion of bilateral common carotid arteries in gerbils. In these reports, the clinical conditions of animals are not clear. The present investigation demonstrated polyribosomal disintegration and dysfunction both during progression of cerebral ischemia and immediately after re-establishment of cerebral circulation. It is noteworthy that disaggregation and dysfunction of polyribosomes occurred regardless of the reversibility of the ischemic process, both after an ischemic period of 30 min and 3 h, and that polyribosomes eventually returned toward normal along with recovery of the clinical condition if the ischemic period was short. Judging from our previous observation, the animals rendered ischemic for 30 min would have been clinically normal 24 h after re-establishment of blood circulation if allowed to live. Kleihues and Hossmann and Cooper et al. did not observe disaggregation of polyribosomes prior to re-establishment of cerebral circulation. This could be due to the difference in experimental models. Nakai et al. demonstrated markedly decreased but not completely diminished cerebral circulation after occlusion of one common carotid artery in symptomatic gerbils. Cooper et al. demonstrated breakdown of polyribosomes to ribosomal subunits after release of intracranial hypertension, while the present work showed disaggregation of the monomer-dimer complex. This was probably due to the difference in the samples for the aggregation profile study, postmitochondrial supernatant vs polyribosomes, and the difference in K+ concentration of the sucrose gradient. Whether they exist as monomers or ribosomal subunits in vivo was difficult to determine even by electronmicroscopic study. There was a difference in sensitivity for polypeptide synthesis between microsomes and polyribosomes. A similar observation has been made in our laboratory in the past with cerebral hypoxia. This may be due to selection artifact during preparation of polyribosomes, or lack of the initiation step with isolated polyribosomes in *in vitro* assay. The mechanism for polyribosomal disaggregation in cerebral ischemia remains uncertain. Possible relationship to ATP level, pH and other factors has been discussed for progressive cerebral ischemia and anoxia. This phenomenon is unlikely to be caused by seizure activity since seizures are not com-

**Table: Effect of Cerebral Ischemia and Re-circulation on Polypeptide Synthesis with Cell-free Systems in vitro**

<table>
<thead>
<tr>
<th>Ischemia Recovery (min) (min)</th>
<th>Microsomes</th>
<th>Polyribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>68.0% ± 15.9 (6)</td>
<td>106.6% ± 29.8 (5)</td>
</tr>
<tr>
<td>60</td>
<td>52.2% ± 11.9 (5)</td>
<td>69.5% ± 15.4 (6)</td>
</tr>
</tbody>
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Results are expressed as percent ± SD of the specific radioactivity (dpm/µg protein or Am unit) of the left cerebral hemisphere from the same animal. The number of experiments are shown in parentheses.
mon during the early stage of cerebral ischemia, and intermittent seizure activity does not affect polypeptide synthesis in this experimental model. Since our previous investigation did not show much effect of pH 5 factor from ischemic tissue, pH 5 factor was prepared only from normal tissue. The mechanism for disaggregation of polyribosomes immediately after re-establishment of cerebral circulation is more puzzling. Interestingly, this phenomenon occurred even when tissue glucose and ATP levels considerably recovered and the tissue lactate level was significantly reduced. This phenomenon is not an artifact due to excessive release of lysosomal ribonuclease since a very prolonged centrifugation or addition of ribonuclease inhibitor made no difference in the aggregation profile, and since our previous investigation did not show excess release of ribonuclease in the early period of cerebral ischemia or anoxia. The findings from the
Electronmicroscopic study is also supportive. Although this study demonstrated swelling of astrocytic processes and we suspect that protein synthesis in glial cells is also affected, judging from our previous investigation, further elucidation of the mechanism for disaggregation of polyribosomes is currently under way in our laboratory. In reversible cases, the nuclear regulatory mechanism for messenger RNA synthesis appears to remain intact to provide messenger RNA for reaggregation of ribosomes.

Clinically, the cellular dysfunction after reestablishment of cerebral circulation may be very important. This situation can follow surgical reestablishment of cerebral circulation after embolec-tomy or anastomosis of superficial temporal artery to middle cerebral artery, and in cerebral anoxia after cardiac arrest. If the cellular dysfunction during recovery process can be altered pharmacologically, it may modify the irreversibility of the process.

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