Emphasized Selective Vulnerability After Repeated Nonlethal Cerebral Ischemic Insults in Rats

Teruyoshi Inoue, MS; Hiroyuki Kato, MD, DMSc; Tsutomu Araki, PhD; and Kyuya Kogure, MD, DMSc

Background and Purpose: We examined the density and distribution of brain damage after repeated periods of nonlethal ischemic insult in rats in comparison with damage after single lethal periods of ischemic insult.

Methods: Transient cerebral ischemia was induced by four-vessel occlusion for 3, 10, 20, and 30 minutes, and 3-minute periods of ischemia were repeated two, three, or five times at 1-hour intervals, followed by 7 days of survival.

Results: Three minutes of ischemia produced no brain damage, but 10–30 minutes of ischemia produced neuronal damage, depending on the length of ischemia, to the selectively vulnerable forebrain regions such as hippocampal CA1 and CA4 subfields, neocortex, striatum, and ventral thalamus, as well as to the brain stem structures (medial geniculate body, substantia nigra, and inferior colliculus) and cerebellar Purkinje cells. Two 3-minute periods of ischemic insult produced neuronal damage to the hippocampal CA1 subfield. Three and five 3-minute insults produced neuronal damage extensively to the selectively vulnerable forebrain areas. An intense cumulative effect of damage was observed in the ventral thalamus, whereas the substantia nigra and the inferior colliculus were resistant to repeated ischemic insults.

Conclusions: Our data indicate that the density and distribution of neuronal damage after repeated ischemic insults are altered as compared with after single ischemia.

KEY WORDS • cerebral ischemia • neuronal damage • rats

Neurons in the brain have different vulnerability to ischemic insult, and specific neuronal populations are preferentially damaged after transient cerebral ischemia.1-6 A number of studies have described the distribution of the selectively vulnerable neurons in the brain. The vulnerable areas encompass the hippocampal CA1 and CA4 subfields, neocortical layers 3 and 5, dorsolateral part of the striatum, ventroposterior part of the thalamus, and the cerebellum. The matter of selective vulnerability has been one of the central issues of postischemic brain damage.

Recently, we reported that brief and morphologically nonlethal cerebral ischemia produces severe neuronal damage to the selectively vulnerable areas when it is induced repeatedly at certain intervals.7-10 Two-minute bilateral carotid artery occlusion in the Mongolian gerbil produces no brain damage. However, two such periods of ischemic insult at a 1-hour interval destroy hippocampal CA1 pyramidal cells, and three or five insults additionally cause extensive neuronal damage to the selectively vulnerable areas, such as the striatum and thalamus.7,9,10 The cumulative damage after nonlethal cerebral ischemic insults has also been described in the rat hippocampus.8

These experimental results showed that even nonlethal cerebral ischemia critically alters the susceptibility of neurons to succeeding ischemic insults. However, it is not fully elucidated whether alterations in the density and distribution of neuronal damage take place when ischemic insults are repeated as compared with single ischemic insults. We therefore compared the density and distribution of neuronal damage after repetitive ischemic insults with those after a single ischemic insult using the four-vessel occlusion model in the rat.11,12

Materials and Methods

Induction of Ischemia

A total of 81 male adult Wistar rats weighing 250–300 g were subjected to four-vessel occlusion11,12 with a minor modification. One day before four-vessel occlusion, the rats were anesthetized with 50 mg/kg i.p. sodium pentobarbital, and both vertebral arteries were electrocoagulated through the alar foramina. The rats were subjected to fasting overnight but allowed free access to water. On the next day, the rats were anesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. A midline cervical skin incision was made, and bilateral common carotid arteries were gently exposed. Needle electrodes were placed under the scalp for electroencephalographic recordings, and 100 units i.p. heparin was injected. Anesthesia was then

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discontinued. When the rats recovered from anesthesia, bilateral common carotid arteries were occluded with aneurysm clips, and the completeness of ischemia was confirmed by isoelectric electroencephalography and a loss of righting reflex. Animals not exhibiting these criteria were discarded. The ischemic insult was terminated by removal of the clips, and restoration of carotid blood flow was verified by direct observation. Animals were divided into groups subjected to single 3-minute (n=6), 10-minute (n=11), 20-minute (n=8), and 30-minute (n=12) occlusions and subjected to two (n=5), three (n=11), and five (n=5) 3-minute occlusions at 1-hour intervals. Six animals underwent sham operation. Body temperature was maintained at close to 37°C with a heating mat and lamp throughout the experiment.

Before the first, third, and fifth 3-minute occlusions, mean arterial blood pressure, arterial pH, PaCO2, PaO2, and plasma glucose level were determined in separate group (n=5). Rectal and cranial temperatures were measured during each repetitive 3-minute ischemic insult (n=5) and during 30-minute ischemia (n=5). Histopathology was not assessed in these animals.

**Histopathology**

Seven days after ischemia, the rats were anesthetized with 50 mg/kg i.p. sodium pentobarbital, and their brains were perfused transcardially with FAM (40% formaldehyde:acetic acid:methanol [1:1:8]) for 20 minutes after a 1-minute heparinized saline flush. The brains were removed, placed in FAM for 2 days, and then embedded in paraffin. Coronal sections 5 μm thick were cut using a microtome and stained with cresyl violet and hematoxylin-eosin. The sections were examined under a light microscope without the examiner knowing the experimental conditions, and the severity of neuronal damage was graded on the following semiquantitative scale: 0, normal; 1, a few neurons damaged; 2, many neurons damaged; and 3, the majority of neurons damaged. The average value of both hemispheres was used for each animal.

**Statistical Analysis**

Mean and standard errors were calculated. Physiological variables were statistically analyzed using repeated-measures analysis of variance followed by Dunnett’s test. Neuronal damage in each of the locations was statistically analyzed using the nonparametric Kruskal-Wallis analysis of variance with a post hoc test using Ryan’s procedure for multiple comparisons. We considered probability values less than 5% significant.

**Results**

Approximately 17% of the animals subjected to ischemia were discarded because of death due to respiratory failure or because of incomplete ischemia as judged by electroencephalographic and behavioral criteria. Behavior of the rats subjected to 10- and 20-minute ischemia and to repeated ischemic insults appeared normal. Five of the 12 animals subjected to 30-minute ischemia died during the recirculation period.

The values for the physiological variables before the first (baseline), third, and fifth 3-minute periods of ischemia are shown in Table 1. Variables other than blood glucose did not differ significantly during the experimental procedure. Blood glucose increased slightly after the first ischemic insult within normal range. Figure 1 shows rectal and cranial temperatures in rats subjected to repeated 3-minute or single 30-minute ischemic insults. In each case, rectal temperatures were maintained at approximately 37°C throughout the experiment. Cranial temperatures fell by 1.0–1.3°C during each repetitive 3-minute ischemic insult; during 10-, 20-, and 30-minute ischemia, the temperatures fell by 2.2°C, 2.7°C, and 3.2°C.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before first ischemia</th>
<th>Before third ischemia</th>
<th>Before fifth ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP (mm Hg)</td>
<td>107±5</td>
<td>104±4</td>
<td>105±5</td>
</tr>
<tr>
<td>pH</td>
<td>7.37±0.03</td>
<td>7.40±0.02</td>
<td>7.40±0.02</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>44±2</td>
<td>46±2</td>
<td>48±3</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
<td>137±6</td>
<td>139±1</td>
<td>139±1</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>129±9</td>
<td>144±8*</td>
<td>148±9*</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td>37.1±0.1</td>
<td>37.0±0.1</td>
<td>37.0±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MABP, mean arterial blood pressure. *p<0.01 different from before first ischemia by repeated-measures analysis of variance and Dunnett’s test.

Table 2 shows the severity of neuronal damage to various regions after single and repeated ischemic insults. Single 3-minute ischemia did not produce morphological changes in any region. Ten-minute ischemia produced neuronal damage to the hippocampal CA1 subfield, hippocampal CA4 subfield, and neocortical layer 3. Twenty- and 30-minute ischemia additionally produced neuronal damage to the dorsolateral striatum, ventral thalamus, medial geniculate body, substantia nigra pars reticulata, inferior colliculus, and the cerebellar Purkinje cells.

**Figure 1.** Rectal (○) and cranial (●) temperatures in five rats subjected to repetitive 3-minute periods of ischemic insult (left) and a 30-minute period of ischemia (right). Values are mean±SEM.
When 3-minute ischemia was repeated two times at 1-hour intervals, moderate neuronal damage occurred to the hippocampal CA1 sector. The parietal cortex and the hippocampal CA4 subfield were also damaged in some of the animals. Three or five 3-minute periods of ischemic insult worsened neuronal damage to these areas and additionally induced neuronal damage to the striatum, hippocampal CA3 subfield, frontal cortex, ventral thalamus, and the medial geniculate body. Three 3-minute insults produced more neuronal damage than did the nearly equivalent 10-minute period of ischemia in the frontal cortex, hippocampal CA3 sector, and the ventral thalamus, but the difference was significant only in the ventral thalamus. In five 3-minute periods of ischemic insult, similar aggravation of neuronal damage was observed in the frontal cortex, parietal cortex, hippocampal CA3 sector, and the ventral thalamus compared with the longer 20-minute period of ischemia. In contrast, the substantia nigra pars reticulata, inferior colliculus, and cerebellar Purkinje cells were insensitive to repeated ischemic insults, and no neuronal damage was observed.

Representative photographs of the thalamus and the inferior colliculus, indicating the regional difference in sensitivity to the repeated ischemic insults, are shown in Figures 2 and 3. In the thalamus, no lesion was detected after a 10-minute period of ischemia, but mild-to-moderate damage was usually observed after three 3-minute periods of ischemic insult. The inferior colliculus was frequently damaged, with eventual formation of phagocytes after a 20-minute period of ischemia. However, no damage was found after five 3-minute periods of ischemic insult.

**Discussion**

The density and distribution of neuronal damage after transient cerebral ischemia has been well documented in both the rat\(^1\text{-}^6,\text{13}\) and the gerbil.\(^14\text{-}^15\) The present results are essentially the same as in the previous reports. The most vulnerable regions were the hippocampal CA1 and the neocortex, followed by the striatum, thalamus, substantia nigra, and inferior colliculus.

Distribution of neuronal damage after repeated non-lethal periods of ischemic insult was generally the same as that after single periods of lethal ischemic insult. However, the major finding of this study is that emphasis of selective vulnerability after repeated ischemic insults was observed in certain regions. The repeated insults aggravated damage to the vulnerable regions at different degrees and changed the pattern of neuronal damage. In particular, as compared with single periods of ischemic insult, the damage was emphasized in the ventral thalamus. Damage to the ventral thalamus after three 3-minute periods of ischemic insult was as severe as that after a single 30-minute period of ischemia, despite the fact that a single 10-minute period of ischemia produced no noticeable neuronal damage. Damage to the thalamus was more severe than that to the striatum after repeated insults; whereas, after a single period of ischemia, damage to the striatum was more severe. The high sensitivity of the ventral thalamus to repeated ischemic insults is compatible with our findings in the gerbil\(^9\) and with the results of Ikeda et al.\(^16\) It may be important that the thalamus, with emphasized vulnerability, was relatively less vulnerable to single periods of ischemia. This suggests that the aggravation of neuronal damage after repeated ischemia is caused by mechanisms other than those which produce neuronal damage after a single period of ischemic insult.

On the other hand, there were regions entirely resistant to repeated ischemic insults; i.e., the substantia nigra, inferior colliculus, and cerebellum. Previous reports using four-vessel occlusion have described the susceptibility of brain stem regions and the cerebellum.\(^3,\text{13}\) The present study also showed a similar result. The inferior colliculus and the substantia nigra were damaged as frequently as the striatum and the thalamus by 20–30 minutes of ischemia. Nevertheless, even after five 3-minute periods of ischemic insult, no damage was

**Table 2. Neuronal Damage After 3, 10, 20, and 30 Minutes of Four-Vessel Occlusion and After Two, Three, and Five 3-Minute Periods of Four-Vessel Occlusion in Rats**

<table>
<thead>
<tr>
<th>Region</th>
<th>Sham (n=6)</th>
<th>3 min (n=6)</th>
<th>10 min (n=11)</th>
<th>20 min (n=8)</th>
<th>30 min (n=7)</th>
<th>2×3 min (n=5)</th>
<th>3×3 min (n=11)</th>
<th>5×3 min (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.73±0.14*</td>
<td>0.63±0.18†</td>
<td>0.71±0.26†</td>
<td>0.00±0.00</td>
<td>1.36±0.26*</td>
<td>1.50±0.52*</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>1.50±0.19*</td>
<td>1.69±0.25*</td>
<td>2.14±0.26*</td>
<td>0.70±0.20</td>
<td>1.77±0.24*</td>
<td>2.40±0.40*</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.14±0.14</td>
<td>1.00±0.39†</td>
<td>2.43±0.43*</td>
<td>0.00±0.00</td>
<td>0.45±0.22</td>
<td>1.10±0.45</td>
</tr>
<tr>
<td>Hippocampal CA1</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>2.32±0.15*</td>
<td>2.75±0.16*</td>
<td>2.64±0.28*</td>
<td>1.60±0.19</td>
<td>2.50±0.19*</td>
<td>2.70±0.30*</td>
</tr>
<tr>
<td>Hippocampal CA3</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.43±0.14</td>
<td>0.31±0.16</td>
<td>0.21±0.15</td>
<td>0.00±0.00</td>
<td>0.91±0.31*</td>
<td>1.30±0.34+</td>
</tr>
<tr>
<td>Hippocampal CA4</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.45±0.13†</td>
<td>0.88±0.18*</td>
<td>0.92±0.17*</td>
<td>0.40±0.19</td>
<td>0.55±0.14*</td>
<td>0.60±0.29†</td>
</tr>
<tr>
<td>Ventral thalamic nucleus</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.44±0.15</td>
<td>1.07±0.44†</td>
<td>0.00±0.00</td>
<td>1.27±0.21††</td>
<td>1.80±0.20+‡</td>
<td></td>
</tr>
<tr>
<td>Medial geniculate body</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.27±0.14</td>
<td>0.69±0.28†</td>
<td>0.64±0.28†</td>
<td>0.00±0.00</td>
<td>0.50±0.19</td>
<td>0.40±0.29</td>
</tr>
<tr>
<td>Substantia nigra pars reticula</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.69±0.37*</td>
<td>1.07±0.44*</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.05±0.05</td>
<td>1.00±0.33*</td>
<td>1.64±0.24*</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Cerebellar Purkinje cells</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.27±0.14</td>
<td>0.50±0.27</td>
<td>0.57±0.30†</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

\(\dagger p<0.05\) and \(\ddagger p<0.01\) different from sham group by nonparametric Kruskal-Wallis analysis of variance with a Ryan post hoc test for multiple comparisons.

\(\ddagger p<0.05\) different from 10-minute ischemia group by nonparametric Kruskal-Wallis analysis of variance with a Ryan post hoc test for multiple comparisons.

\(\dagger p<0.05\) and \(\ddagger p<0.01\) different from 20-minute ischemia group by nonparametric Kruskal-Wallis analysis of variance with a Ryan post hoc test for multiple comparisons.
FIGURE 2. Photomicrographs of ventral thalamic nucleus in rat 7 days after transient ischemia. Panels a and b: Animals subjected to a single 10-minute period of ischemia exhibited no neuronal damage. Panels c and d: Scattered eosinophilic neurons appear in rats after three 3-minute periods of ischemic insult. Hematoxylin-eosin stain. Bar, 100 μm.
FIGURE 3. Photomicrographs of inferior colliculus in rat 7 days after transient ischemia. Panels a and b: Vacuolization and dense infiltration of macrophages are found after 20-minute period of ischemia. Panels c and d: Neurons are intact after five 3-minute periods of ischemic insult. Hematoxylin-eosin stain. Bar, 100 μm.
found in the brain stem structures and the cerebellar Purkinje cells. The lack of damage to the brain stem structures is inconsistent with the results in gerbils.5,10 The difference in severity of ischemia may produce the contradiction because four-vessel occlusion in rats produces severe ischemia in the forebrain but moderate ischemia in the lower brain structures.17-20 However, reduction in blood flow in the midbrain in the gerbil is severe20; therefore, the difference in the reduction of blood flow may explain the different brain stem damage in rats and gerbils. The resistance of brain stem structures may explain the low mortality rate of rats subjected to repeated ischemic insults, despite the fact that forebrain structures are similarly damaged.

The cumulative damage in the forebrain after nonlethal ischemic insults is a common finding in both rats and gerbils. This phenomenon suggests that even nonlethal ischemia alters the susceptibility of neurons to succeeding ischemic insults. However, the underlying pathomechanisms of the cumulative effect remain to be elucidated.

The possible role of hypoperfusion was suggested by reports that 5 minutes of ischemia in gerbils produced a pronounced cumulative effect on edema and tissue injury when induced repeatedly during the period of marked posts ischemic hypoperfusion.21-23 Because even nonlethal ischemia can produce the hypoperfusion that is most prominent 1 hour after ischemia,20 posts ischemic hypoperfusion may play an essential role in the cumulative effect. Because the degree of hypoperfusion is proportional to the severity of the initial ischemic insult,17,24 the posts ischemic hypoperfusion may be less pronounced in the brain stem and the cerebellum in the rat, which may explain the insensitivity of the brain stem and Purkinje cells to repeated insults in the rat.

We have also demonstrated that even nonlethal ischemia produces a severe inhibition of protein synthesis in the selectively vulnerable areas during the early stage of recirculation in gerbils,25 which suggests that incomplete recovery from metabolic disturbance is one of the causes of neuronal damage after repeated insults. Of interest is that a clear retardation of the recovery of protein synthesis was found in the hippocampal CA1 sector25 that is most vulnerable not only to single periods of ischemia but also to repeated nonlethal ischemic insults, whereas extensive recovery of protein synthesis was observed in the neocortex, the striatum, and the thalamus. Therefore, the metabolic disturbance may account for the cumulative damage by the repeated nonlethal insults but not for the emphasis of susceptibility on the thalamus.

In interpreting the greater damage after repeated insults in this experiment, we must point out the possibility that the greater cerebral hypothermia in lethal ischemia may ameliorate neuronal damage. As a previous report has shown,26 the maintenance of rectal temperature failed to prevent the decrease of cranial temperature in rats subjected to ischemia. The cranial temperature fell by 2-3°C during 10-30 minutes of ischemia, whereas in cases of repeated ischemic insults the temperature fell in smaller degree during each 3-minute period of ischemia (Figure 1). Recent studies demonstrated that a drop of only 2°C in brain temperature attenuates ischemic damage in vulnerable regions.27,28 Cerebral hypothermia, therefore, may lessen neuronal damage in longer lethal ischemia. However, hypothermia alone cannot explain the modified pattern of vulnerability after repeated ischemic insults because it is not likely that brain hypothermia occurs differently among regions in concert with the different degrees of neuronal damage.

In conclusion, we demonstrated that repeated nonlethal ischemic insults in the rat produce neuronal damage to the selectively vulnerable forebrain areas but not to the brain stem regions and that an intense cumulative effect was found on the damage to the ventral thalamus. Thus, repeated ischemic insults change the pattern of neuronal damage as compared with that in a single period of ischemia. These findings are important in elucidating neuronal vulnerability to ischemia. Further studies using this model will be needed to clarify the pathomechanisms and dynamics of ischemic neuronal injury.

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