Poor Recovery of Mitochondrial Redox State in CA1 After Transient Forebrain Ischemia in Gerbils

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Background and Purpose—Several investigations have detected evidence of apoptosis in delayed neuronal death, but controversy prevails regarding this point. Recent studies have implicated mitochondria in apoptotic events. To explore relationships between delayed neuronal death and dysfunction of the respiratory chain, we analyzed mitochondrial redox changes in the gerbil hippocampus.

Methods—We assessed the mitochondrial redox state in gerbil hippocampus before, during, and at various time points after 5 minutes of forebrain ischemia. The redox state was examined with a low-temperature fluorometer. Fluorescence signals of flavoprotein and NADH were measured, and their fluorescence ratio was calculated as a mitochondrial redox ratio (MRR) equal to flavoprotein/(flavoprotein + NADH).

Results—Ischemia increased NADH and decreased flavoprotein signals in all hippocampal areas, but reduction in MRR was greater in CA1 than in other areas of the hippocampus. Immediately after recirculation, MRR recovery was delayed in the CA1 and the dentate gyrus, and the reduction in MRR persisted in CA1.

Conclusions—These results suggest that during ischemia CA1 experiences more pronounced hypoxia (state V) than less vulnerable regions. Persistent MRR reduction in CA1 is attributed to dysfunction of the electron transport system, and this phenomenon may be importantly involved in apoptosis. (Stroke. 1998;29:2421-2425.)

Key Words: apoptosis ■ cerebral ischemia ■ mitochondria ■ neuronal death ■ gerbils

Neuronal death in certain areas is a well-known outcome after transient cerebral ischemia.1-3 A peculiar form of cell death termed delayed neuronal death is seen in the CA1 region of the hippocampus. Since blood flow and energy metabolism completely recover during recirculation after 5 minutes of forebrain ischemia,4-6 delayed neuronal death has been attributed not to energy failure but to free radicals,7-9 excitatory amino acids,10 disordered protein metabolism,11-13 or apoptosis.14-17 On the other hand, mitochondrial dysfunction has been reported after recirculation.18-26 Recent studies have postulated that apoptosis or programmed cell death is closely linked to mitochondrial dysfunction. Reduction in mitochondrial transmembrane potential or release of cytochrome c from the outer surface of the inner mitochondrial membrane into the cytosol is thought to be an early event in the apoptotic process.27-33 Therefore, we investigated mitochondrial redox changes in the hippocampus in detail during ischemia and after recirculation, finding differences in redox changes between CA1 and less vulnerable hippocampal subregions.

Materials and Methods

Animal studies were performed under a protocol approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee. A total of 41 Mongolian gerbils from Tumble-brook Farms (West Brookfield, Mass) weighing 60 to 80 g were used. All animals were maintained in a 12/12-hour light/dark cycle. Food and water were available ad libitum until the beginning of the experiment. Five minutes of forebrain ischemia was induced by occlusion of both common carotid arteries under a 2% halothane inhalation mixture with 30% O2 and 68% N2O. After anesthesia the entire body, including the head, was warmed with a heating pad to maintain the rectal temperature at 37±0.5°C. The brain then was frozen, and redox state was measured before (n=6) and during ischemia (n=5), immediately after recirculation (n=3), and at 1 (n=4), 6 (n=4), and 12 hours (n=4) after recirculation and at 1 (n=3), 2 (n=3), 3 (n=3), 4 (n=3), and 7 (n=3) days after recirculation. The brains were frozen according to the method developed by Pontén et al.,34 using a funnel and liquid nitrogen. Endotracheal intubation was performed, and a funnel with an opening of ~1 cm was mounted onto the skull. To prevent respiratory obstruction and rib cage freezing caused by leakage of liquid nitrogen, the bottom of the funnel was covered by pulling up the scalp and sealing the opening with a solution containing ethanol and glycerin. Mechanical ventilation was begun just before the start of freezing. The respirator was adjusted so that approximate end-tidal CO2 concentrations were within the range of 35 to 40 mm Hg determined with the use of Respina IH26 equipment (NEC Medical Systems). After completion of the freezing procedure, the entire body was soaked in liquid nitrogen, and the head was severed with an electric saw. Sections of the scalp and rib cage freezing caused by leakage of liquid nitrogen, the bottom of the funnel was covered by pulling up the scalp and sealing the opening with a solution containing ethanol and glycerin. For measurement of redox state at 5 minutes of ischemia, funnel freezing started at 4 minutes and ended at 6 minutes after induction of ischemia.

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The redox state of the brain was measured with a low-temperature fluorometer developed in our laboratory. Since the reduced form of NADH (pyridine nucleotide) is fluorescent and the oxidized form (NAD\(^+\)) is not, measurement of NADH fluorescence is often used for estimating the intracellular redox ratio. However, the signal intensity of fluorescence depends on the concentration of NADH and is further altered by light-absorbing substances such as hemoglobin. To partially offset such effects, we also measured the fluorescence of the oxidized flavin proteins (flavoprotein) as an electron receptor equivalent to the fluctuations of NAD\(^+\), and we compared their fluorescence with NADH fluorescence as a mitochondrial redox ratio (MRR) using the equation \[ \text{MRR} = \frac{\text{flavoprotein}}{\text{flavoprotein} + \text{NADH}}. \] The method of redox scanning has been described in detail elsewhere. 35 Briefly, while the brain was frozen with liquid nitrogen, the hippocampus was exposed by shaving off the brain in coronal planes to 3 mm posterior to the bregma. For the measurement of NADH, the excitation and emission wavelengths were 366 and 450 nm, respectively, and for the measurement of flavoprotein, the excitation and emission wavelengths were 436 and 520 nm, respectively. With the use of a glass fiber 25 \(\mu\)m in diameter, excitation light corresponding to NADH or flavoprotein was channeled alternately onto the surface of specimen. The resulting fluorescence signals of NADH and flavoprotein were delivered by another glass fiber to a photomultiplier tube to measure fluorescence.35,36 The step width was 20 \(\mu\)m, and the matrix was 128 steps long and 256 steps wide. Both flavoprotein and NADH fluorescence signals were converted to 1 of 256 values (from 0 to 255) with an analog-digital converter and then displayed with a 16-grade gray-scale value corresponding to the intensity of fluorescence. On the basis of the fluorescence signals of flavoprotein and NADH, the MRR of each matrix, which could range from 0 to 1, was calculated as an indicator of the mitochondrial redox state. The MRR also was displayed as a 16-grade gray-scale value: the smaller the MRR, the darker was the area (reduction), while conversely, the higher the MRR, the lighter was the area (oxidation).

**Results**

Ischemia increased the fluorescence signal of NADH and decreased that of flavoprotein. The degree of change was greater for NADH. A strong flavoprotein fluorescence band was seen in the granule cell layer of the dentate gyrus and in the pyramidal cell layer from the CA1 to CA3 regions; the fluorescence signal of flavoprotein in the pyramidal cell layer of the CA3 region was especially strong. Although the intensity of fluorescence that originated from flavoprotein in these areas was reduced somewhat by ischemia, the degree of change was smaller than in other areas. The fluorescence signals of flavoprotein or NADH were nonexistent or extremely weak in the cerebral ventricles and blood vessels. Changes in local redox state were detected more easily by MRR images than by flavoprotein or NADH images.

**Findings Before Ischemia**

On MRR images, the pyramidal cell layer and the granule cell layer of the dentate gyrus were seen as white bands. No regional differences were evident in MRR among the stratum oriens, stratum radiatum, and the molecular layer of the dentate gyrus (Figure 1).

**Findings During Ischemia**

Ischemia decreased the fluorescence signal of flavoprotein and increased that of NADH. The intensity of the fluorescence signal of NADH was particularly high in the stratum oriens and stratum radiatum of the CA1. As a result, the decrease in MRR in these areas was greater than in other areas. Decreases in MRR in these areas were heterogeneous and seen as spots. Decreases in MRR in the CA1 region were larger medially than laterally (Figure 1).

**Findings After Recirculation**

Immediately after recirculation, MRR recovered more slowly in the hippocampus than in the cortex. MRR in the stratum oriens and the stratum radiatum of CA1 and the molecular layer of the dentate gyrus recovered less completely than in other areas. Unlike those seen during ischemia, decreases in MRR were homogeneous (Figure 1).
MRR recovered poorly in CA1, and the degree of decrease in MRR was especially remarkable 12 hours after recirculation. Except for MRR in CA1, MRR in the hippocampus had returned to preischemic levels 1 hour after ischemia. Three days after ischemia the fluorescence signal of flavoprotein increased and that of NADH decreased in the subiculum and the medial portion of the CA1 region (Figure 1). Reflecting these changes, the MRR in these areas increased strongly. This phenomenon extended laterally over time, and by day 7 the entire CA1 region was bright on MRR images (Figure 1). MRR changes of the stratum radiatum in the CA1 and CA3 regions are shown as percentages of control MRR in Figure 2. The degree of MRR reduction in CA1 during ischemia was 50.8%, which is significantly larger than the 23.6% reduction seen in CA3 (P<0.05, Mann-Whitney U test). Significant MRR reduction also was observed in CA1 at 6 and 12 hours after recirculation (P<0.05, Mann-Whitney U test).

Discussion
Since NADH is an intrinsic fluorochrome, several investigations have used this compound to measure the redox state of various tissues. However, no reports have closely examined evolution of delayed neuronal death in the hippocampus from the viewpoint of changes in redox state. Using a narrow glass fiber 25 μm in diameter, we were able to observe redox changes in the hippocampus of gerbils. To minimize the effect of the concentrations of fluorochromes and light-absorbing substances such as hemoglobin, the fluorescence signal of oxidized flavoprotein, another natural intrinsic fluorochrome, was measured simultaneously.

Ischemia decreased the fluorescence signal of flavoprotein, increased that of NADH, and decreased MRR, suggesting that the rate of internal respiration was limited by oxygen rather than by substrates in ischemia. Interestingly, the degree of reduction in MRR in the ischemic period was marked in CA1. Previous studies have shown no regional difference in cerebral blood flow between vulnerable and invulnerable regions during ischemia and reperfusion. Nonetheless, from the viewpoint of redox analysis, hypoxia (low MRR) was more prominent in CA1 than in other areas of the hippocampus in these periods. Although capillary density in CA1 has been found to be lower than in other hippocampal areas, some conflicting results have been reported. The exact mechanism underlying the prominent reduction of the MRR in CA1 remains unclear, but our results suggest that pyramidal cells in CA1 experienced more profound hypoxia than those in other less vulnerable regions during 5 minutes of forebrain ischemia.

Cerebral blood flow and glucose metabolism are thought to return to preischemic levels after 5 minutes of forebrain ischemia in all areas, including CA1. However, in the present study the MRR in CA1 did not completely recover after recirculation. Reductions in MRR usually are seen in mitochondria in state V or in a state of hypoxia. Most likely, this conflicting result reflects inhibition of electron flow in the respiratory chain. Electron carriers that function at points preceding the inhibited step would become fully reduced, and those that function at points after the block would be completely oxidized. In the present study MRR reduction occurred when electron flow was blocked at carriers following ubiquinone. Abe et al recently reported that cytochrome c oxidase dysfunction becomes evident in CA1 soon after recirculation. More recently, close links between mitochondrial dysfunction and apoptosis have been postulated. One could hypothesize that the poor recovery of the mitochondrial redox state in CA1 after recirculation may result from cytochrome c release from the outer surface of the inner mitochondrial membrane, resulting in electron stagnation in the respiratory chain.

MRR strongly increased in CA1 4 days after recirculation. This phenomenon may be caused by (1) a shift to state III; (2) injury to the inner mitochondrial membrane; ie, uncoupling of internal respiration; or (3) phagocytosis resulting in strong oxidative reactions affecting flavoprotein. The shift to state III is most likely not responsible, because nerve cells in CA1 begin to die 4 days after recirculation. Since the timing and site of onset of the abnormal increase in MRR match those of morphological changes in nerve cell death, this abnormal increase of MRR seems to correlate with elimination of dead nerve cells.

Contamination with nonspecific fluorescence signals and incomplete coupling between flavoprotein and NAD+/NADH may represent drawbacks of the present method. In our study a strong zonal flavoprotein fluorescence signal was detected in the pyramidal layer and granule cell layer of the dentate gyrus, suggesting the presence of analogous fluorochromes or fluorescence signals of flavoprotein that do not match redox state. However, when NADH signal changes are considered, the NADH signal was stronger in CA1 than in other areas during ischemia, and this tendency persisted after recirculation. This suggests that the degree of hypoxia during ischemia may be stronger in CA1 than in other areas. Delayed neuronal death has been differentiated from the neuronal death that results from acute energy failure seen in complete
and controversy continues as to whether delayed neuronal death represents apoptosis. Nonetheless, we believe that the prolonged reduction of MRR occurring with recirculation may result from partial mitochondrial dysfunction that may induce apoptosis in a slow process of neuronal death, as is seen in delayed neuronal death.

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References
Editorial Comment

It is well known that the CA1 region of the hippocampus is especially vulnerable to ischemia. The reasons for this increased vulnerability have not been fully clarified.

In the accompanying article, Shiino and colleagues followed the redox state of mitochondria by measuring the fluorescence of oxidized flavoprotein and of NADH in the hippocampus during and after a brief episode of ischemia. As expected, they found increases in NADH fluorescence and decreases in flavoprotein fluorescence during ischemia. However, the increases in NADH were more pronounced in the CA1 region than in other areas of the hippocampus. Surprisingly, these changes persisted after recirculation for several days. Since after such a brief period of ischemia, blood flow is reestablished within minutes following recirculation, it is unlikely that the changes in redox state in the CA1 region can be explained by persistent hypoxia. It is more likely that they are due to an interruption in the function of mitochondria, so that the normal flow of electrons through the electron transport chain is interrupted.

The importance of these findings is that it directs attention to the mitochondrial changes that occurred during ischemia as the most likely explanation for the high vulnerability of the CA1 regions to ischemia. More work will be necessary to identify the specific features of the mitochondria in this region that may render them more vulnerable to damage from ischemia.

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