Reduction of Apurinic/Apyrimidinidic Endonuclease Expression After Transient Global Cerebral Ischemia in Rats
Implication of the Failure of DNA Repair in Neuronal Apoptosis

Makoto Kawase, MD; Miki Fujimura, MD; Yuiko Morita-Fujimura, MS; Pak H. Chan, PhD

Background and Purpose—To clarify the relationship between apurinic/apyrimidinidic endonuclease (APE/Ref-1), a multifunctional protein in the DNA base excision repair pathway, and delayed neuronal cell death associated with apoptosis, we examined the expression of APE/Ref-1 before and after transient global ischemia in rats.

Methods—Global ischemia was induced by bilateral common carotid artery occlusion and hypotension. Expression of the APE/Ref-1 protein was evaluated by Western blot and immunohistochemical analyses. Apoptosis after global ischemia was observed by DNA electrophoresis and terminal deoxynucleotidyl transferase–mediated uridine 5′-triphosphate–biotin nick end labeling (TUNEL) staining.

Results—Immunohistochemistry showed the nuclear expression of APE/Ref-1 in the control brains. Nuclear immunoactivity of APE/Ref-1 was significantly decreased 2 days after 10 minutes of ischemia in the hippocampal CA1 subregion. Western blot analysis of a sample from the normal brains showed a characteristic 37-kDa band, which was reduced in the hippocampal CA1 subregion after ischemia. A significant amount of DNA fragmentation was observed at 3 days but not at 1 day after ischemia. Double staining with APE/Ref-1 and TUNEL clearly showed that the neurons that lost APE/Ref-1 immunoreactivity became TUNEL positive.

Conclusions—Our data provide evidence that APE/Ref-1 decreased in hippocampal CA1 neurons after transient global ischemia and that this reduction precedes DNA fragmentation, which is destined to cause apoptosis. Our results suggest the possibility that a decrease of APE/Ref-1 activity and the failure of DNA repair may underlie the mechanism of apoptosis after transient focal ischemia. (Stroke. 1999;30:441-449.)

Key Words: DNA base excision repair ■ apoptosis ■ cerebral ischemia, transient ■ rats

The DNA repair enzyme apurinic/apyrimidinidic endonuclease (APE/Ref-1) is a multifunctional protein in the DNA base excision repair (BER) pathway that is responsible for repairing apurinic/apyrimidinidic (AP) sites in DNA.1 DNA BER is assumed to require 2 types of enzymes, such as DNA glycosylases and APEs.2-5 DNA glycosylases remove a damaged base, which could be caused by various kinds of insults including oxidative stress, creating an AP site in the DNA that is then acted on by an APE/Ref-1.3,5 Then the repair is completed by abasic residue followed by synthesis of a new base by DNA polymerase and ligation. Incomplete repair of AP sites is reported to cause mutagenesis and genetic instability.6 Although little is known about the relationship of the DNA BER pathway to necrosis, apoptosis, or both in neuronal cells, recent evidence suggests that down-regulation of APE/Ref-1 expression is associated with apoptosis in cells of the myeloid lineage.7

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Transient global cerebral ischemia is an attractive model for analyzing selective vulnerability in the hippocampal CA1 subregion. Neuronal death in the CA1 subregion after global ischemia has been shown to occur in a delayed fashion8 and undergoes apoptotic cell death, in part.9,10 We have shown that reactive oxygen species (ROS), superoxide anion in particular, is an important factor for the development of DNA damage after transient global ischemia in mice and rats that overexpress superoxide dismutase (SOD).11-13 In a study of mutagenesis, Liu et al14 suggested that free radicals could attack the nuclear genes and cause genetic instability after mouse forebrain ischemia. Growing evidence suggests that the involvement of DNA damage and repair in cell death mechanisms underlies stroke and cerebral trauma.15 Nuclear translocation of DNA repair enzymes such as poly(ADP-ribose) polymerase (PARP) and proliferating cell nuclear...
antigen from the cytosol has been shown after transient cerebral ischemia. Furthermore, Gillardon et al. have reported that APE/Ref-1 mRNA expression was increased after transient global ischemia induced by cardiac arrest. These data suggest the role of these enzymes in repairing DNA damage after ischemia/reperfusion. Therefore, we sought to clarify the relationship between the expression of the APE/Ref-1 protein and apoptotic cell death in delayed neuronal cell damage after transient global ischemia. In the present study, a significant amount of DNA fragmentation was detected by both DNA gel electrophoresis and terminal deoxynucleotidyl transferase–mediated uridine 5′-triphosphate-biotin nick end-labeling (TUNEL) 3 days after ischemia. Using immunohistochemical and Western blot analysis on the same ischemia/reperfusion model, we examined APE/Ref-1 expression after transient global ischemia. We also investigated the relationship between loss of APE/Ref-1 expression and DNA damage using double staining. Our results suggest the possibility that the DNA repair mechanism may contribute to delayed neuronal cell death after transient global cerebral ischemia.

Materials and Methods

Global Cerebral Ischemia

Ten minutes of transient global ischemia was induced by bilateral common carotid artery occlusion and bleeding to lower the mean arterial blood pressure to 35 to 40 mm Hg, using the method originally described by Smith et al. with some modifications. Male rats (275 to 325 g, Charles River Laboratories, Wilmington, Mass) were anesthetized with 5% isoflurane and maintained during surgery at a level of 1.0% to 2.0% isoflurane in 70% N2 O and 30% O2 under spontaneous breathing. The rectal temperature was controlled at 37.0±0.5°C during surgery with a feedback-regulated heating pad. The femoral artery was exposed and catheterized with a PE-50 catheter to allow continuous recording of the arterial blood pressure and removal of blood samples for blood gas analysis. Blood flow in the forebrain, measured by laser Doppler flowmetry, dropped to 10% of the baseline level during ischemia and rapidly returned to the preischemic level after reperfusion. After recovery of the arterial blood pressure, the arterial blood was collected for blood gas analysis. After regaining consciousness, the animals were maintained in an air-conditioned room at 20°C. All animals were treated in accordance with Stanford University guidelines and the animal protocol approved by Stanford University’s Administrative Panel on Laboratory Animal Care.

Gel Electrophoresis

Animals were euthanized 3 days after 10 minutes of global ischemia/reperfusion. Thirty to 50 mg wet weight of tissue was taken from the hippocampal CA1 subregion after the brain was cut coronally. Samples were incubated overnight in 0.6 mL lysis buffer (0.5% sodium dodecyl sulfate [SDS], 10 mM Tris–HCl and 0.1 mM EDTA) with 0.6 mg proteinase K (Boehringer-Mannheim, Indianapolis, Ind) at 55°C. The DNA was extracted with equal volumes of phenol and phenol-chloroform-isooamyl alcohol (25:24:1) and precipitated overnight in 0.2 mol/L sodium chloride in 100% ethanol at −80°C. The DNA was washed 2 times with 75% ethanol, air-dried, and resuspended in DNase-free water (Sigma, St. Louis, Mo). The DNA concentration was measured by using To-Pro-1 dye (Molecular Probes, Eugene, OR). Gel electrophoresis for detecting DNA laddering was performed according to the manufacturer’s instructions (Trevigen, Gaithersburg, Md). One microgram of DNA was incubated with 50 μg/mL of DNase-free RNase (Boehringer-Mannheim) for 30 minutes at 37°C. Then the samples were reacted with Klenow enzyme (Trevigen) and dNTP (Trevigen) for 10 minutes at room temperature. Samples were mixed with a loading buffer and subjected to electrophoresis on 1.5% agarose gel. Then the gel was washed with 0.25 mol/L HCl, 0.4 mol/L NaOH/0.8 mol/L NaCl, and 0.5 mol/L Tris buffer (pH 7.5). DNA was transferred to a nylon membrane overnight in 10× SSC (0.01 mol/L sodium chloride, 60 mMol/L sodium citrate, pH 7.4). The membrane was first blocked by 5% powdered milk (Bio-Rad, Hercules, Calif) in 0.1 mol/L phosphate-buffered saline (PBS) for 30 minutes, and incubated with Strept–horseradish peroxidase conjugate (Trevigen) for 30 minutes. Finally, the bands were visualized by chemiluminescence method with PeroxyGlow (Trevigen), and the films were exposed to x-ray film.

Histological Assessment

Anesthetized animals were perfused with 10 U/mL heparin and subsequently with 4% formaldehyde in PBS, pH 7.4, 1, 2, and 3 days after reperfusion following ischemia. Brains were removed, post-fixed for 24 hours in 4% formaldehyde and sectioned at 50 μm on a vibratome. For histological assessment of neuronal damage, the brain sections were stained with cresyl violet. To clarify that the cells in the pyramidal cell layer were neurons, immunohistochemical analysis with anti-microtubule–associated protein (MAP) was performed. Sections were incubated with 3% H2O2 in PBS and 20% normal horse serum and exposed to anti-MAP2 monoclonal antibody (1:1000; RPN 1194; Amersham International, Buckinghamshire, England) in PBS for 3 days at 4°C. The slides were rinsed and incubated with biotin-conjugated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, Calif) for 30 minutes, rinsed, and then incubated with avidin-biotin–horseradish peroxidase solution (ABC kit, Vector Laboratories) for 30 minutes. Then sections were visualized using 0.025% 3,3′-diaminobenzidine hydrochloride (DAB) and 0.075% H2O2 in PBS. The slides were rinsed with water, stained with methyl green for 10 minutes, dehydrated, and mounted.

In Situ Labeling of DNA Fragmentation

The experimental animals were euthanized at 1, 2, and 3 days after 10 minutes of global ischemia/reperfusion. The brains were removed, rapidly frozen in −20°C 2-methylbutane, −50°C dry ice, and stored at −80°C. They were sectioned with a cryostat into a thickness of 20 μm. Frozen brain sections at the level of the hippocampus were stained using an in situ technique (TUNEL reaction) to detect the DNA-free 3′-OH ends as described. Briefly, frozen brain sections were fixed for 30 minutes in 3.7% formaldehyde in PBS, pH 7.4. The slides were placed in 1× terminal deoxynucleotidyl transferase (TdT) buffer (Life Technologies, Gaithersburg, Md) for 15 minutes, reacted with TdT enzyme (Life Technologies), and biotinylated with 16-dUTP (Boehringer-Mannheim) at 37°C for 60 minutes. The slides were then washed in 2× SSC (150 mmol/L sodium chloride, 15 mmol/L sodium citrate, pH 7.4) for 15 minutes, and washed in PBS 2× for 15 minutes. ABC solution (Vector Laboratories) was applied to the sections for 30 minutes, then the slides were washed for 15 minutes with 0.175 mol/L sodium acetate. Staining was visualized using 0.025% DAB and 0.075% H2O2 in PBS with 0.4 mg/mL nickel sulfate. The slides were rinsed with water, stained with methyl green for 10 minutes, dehydrated, and mounted.

Western Blot Analysis

Whole-cell protein extraction was performed. Samples from the bilateral hippocampal CA1 subregion, striatum, and cortex were cut into pieces at 3 days after reperfusion and put into 10× volume of Tris-glycine-SDS sample buffer (Novex, San Diego, Calif). Samples were then gently homogenized by douncing 20× in a Teflon homogenizer (Wheaton, Millville, NJ). Equal amounts of the samples (10 μL) were loaded per lane. The primary antibodies were either 1:1000 dilution of polyclonal antibody against APE/Ref-1 (Novus Biologicals, Littleton, CO) or 1:10 000 dilution of anti-β-tubulin monoclonal antibody (Sigma). For APE/Ref-1 detection, Western blot analysis was performed with horseradish peroxidase–conjugated anti-rabbit IgG using the Boehringer-Mannheim chemi-
luminescence system. Recombinant human APE/Ref-1 was a generous gift from Novus Biologicals. As the internal control, Western blot analysis of β-tubulin was performed with horseradish peroxidase–conjugated anti-mouse IgG reagents (Amersham International).

**Immunohistochemistry of APE/Ref-1**

Sections fixed by transcardiac perfusion with 4% formaldehyde were made as histological assessments. The sections were incubated with blocking solutions as described21 and reacted with anti-apurinic/apyrimidinic endonuclease polyclonal antibody (Novus Biologicals) at a dilution of 1:100. Immunohistochemistry was performed using the ABC kit (Vector Laboratories) and visualized with 0.025% DAB and 0.075% H2 O2 in PBS. Then the nuclei were counterstained with methyl green solution for 10 minutes. As a negative control, sections were incubated without primary antibodies.

**Double-Labeling with APE/Ref-1**

**Immunohistochemistry and TUNEL**

To clarify the spatial relationship between APE/Ref-1 expression and DNA damage, we performed double staining of APE/Ref-1 antibody and TUNEL as previously described,22 with minor modifications. After transcardiac perfusion, fixed sections were immunostained with APE/Ref-1 antibody as described above, the sections were mounted on glass slides (Superfrost; Fisher Scientific, Pittsburgh, Pa), passed through ethanol (70%, 95%, 100%, and 100%), and then immersed in chloroform for 5 minutes. The sections were rehydrated by passage through a decreasing ethanol series and rinsed in PBS. After eliminating peroxidase activity with 3% H2 O2 in PBS, TUNEL was performed as described above. The slides were rinsed with water, stained with methyl green, dehydrated, and mounted.

**Quantification and Statistical Analysis**

The immunoreactive cells and TUNEL-positive cells in the hippocampal CA1 subregion were quantified with a light microscope by a blinded investigator. A 5×5-mm grid was located approximately at the center of the CA1 subregion. The ratio of the number of APE/Ref-1-immunopositive cells and TUNEL-positive cells to the total number of cells in the grid was calculated and expressed as percent of immunopositive and TUNEL-positive cells in each group. The quantitative analysis of these cells was evaluated using factorial ANOVA between each group. Significance between groups was assigned at P<0.05.

**Results**

**Physiological Data**

No animals died during the operation or after reperfusion. Physiological parameters are shown in the Table. Mean arterial blood pressure was significantly increased after reperfusion. Other parameters were not significantly different 10 minutes before or after global ischemia (Table).

**DNA Gel Electrophoresis**

To confirm the nucleosomal DNA fragmentation, we analyzed DNA from the hippocampal CA1 subregion and the cortex. DNA laddering was absent in the control tissue (Figure 1, lanes 1 and 2). A significant amount of DNA laddering was detected in the hippocampal CA1 subregion 3 days after ischemia (Figure 1, lane 3) but not in the cortex (Figure 1, lane 4).

**Histopathological Analysis of Hippocampal Injury**

Cresyl violet staining showed the shapes of cells in the hippocampal CA1 pyramidal cell layer (Figure 2A, 2B) and MAP2 immunohistochemistry showed neuronal dendrites of the cells (Figure 2E, 2F) in the control brains. These data suggest that almost all cells in the pyramidal cell layer were neurons. Two days after global ischemia, some scattered cells in the pyramidal cell layer underwent ischemic change (Figure 2C), such as a triangle-shaped shrunken nucleus (Figure 2D). The TUNEL reaction showed DNA damage in the nucleus 2 days after global ischemia (Figure 2H). The TUNEL-positive cells were restricted to the pyramidal cell layer in the CA1 subregion (Figure 2G).

<table>
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<tr>
<th>Physiological Parameters</th>
<th>pH</th>
<th>PCO2, mm Hg</th>
<th>PO2, mm Hg</th>
<th>MABP, mm Hg</th>
</tr>
</thead>
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<tr>
<td>Preischemic</td>
<td>7.399±0.044</td>
<td>45.58±4.45</td>
<td>128.33±19.61</td>
<td>100.00±7.07</td>
</tr>
<tr>
<td>Postischemic</td>
<td>7.346±0.034</td>
<td>48.25±4.27</td>
<td>124.93±7.36</td>
<td>125.00±10.80*</td>
</tr>
</tbody>
</table>

All values shown are mean±SD (n=4). MABP indicates mean arterial blood pressure.

* A significant difference was observed between preischemic and postischemic MABP status by factorial ANOVA (P<0.01).
Western Blot Analysis of APE/Ref-1 Protein Expression After Transient Global Ischemia

As shown in Figure 3, APE/Ref-1 immunoreactivity was evident as a single band of molecular mass 37-kDa whole-cell fraction in the control brain (H, hippocampal CA1 subregion; S, striatum; C, cortex; upper panel) and is significantly decreased in the hippocampal CA1 subregion but remains the same in the striatum and cortex 3 days after 10 minutes of global ischemia. On the contrary, a consistent amount of β-tubulin immunoreactivity is shown in the lower panel.

Constitutive Expression of APE/Ref-1 in Normal Adult Rat Brains

The APE/Ref-1 protein was constitutively expressed in the hippocampus of the normal rat brains. It was mainly expressed in the nucleus, which is consistent with the previous report. We observed regional predominance in the hippocampus (Figure 4B, 4D) compared with the cortex and striatum (data not shown). One day after 10 minutes of ischemia and reperfusion, no remarkable change was observed (Figure 4C). A moderate reduction of nuclear APE/Ref-1 immunoreactivity was observed in hippocampal CA1 neurons 2 days after ischemia, and some scattered neurons of the CA1 pyramidal cell layer showed ischemic changes (Figure 4F). At 3 days after ischemia, the APE/Ref-1–positive cells were significantly decreased in the CA1 subregion, and almost all neurons had shrunk and degenerated (Figure 4G). There was no immunoreactivity in the control brain slices, which were treated without a primary antibody (Figure 4A).

Double Labeling with APE/Ref-1 Expression and DNA Fragmentation Detected by TUNEL Staining After Ischemia

As shown in Figure 5A, double labeling with APE/Ref-1 immunoreactivity and TUNEL staining clarified the spatial relationship between loss of APE/Ref-1 expression and DNA fragmentation in the hippocampal CA1 subregion 2 days after ischemia. Three types of CA1 cells were observed. The first type of cell still had APE/Ref-1 immunoreactivity (arrows) that was associated with 2 different expression patterns of APE/Ref-1; 1 was the same pattern as the control and the other had a concentrated expression in the nucleus, which shrank moderately. The second type had shrunken nuclei stained by methyl green but had neither APE/Ref-1 immunoreactivity nor TUNEL reactivity. The third type of cells was TUNEL-positive (arrowhead). No cells were observed to have both APE/Ref-1 immunoreactivity and TUNEL reactivity.
Time Course of Reduction in APE/Ref-1 Expression and Increase in Fragmented DNA Cells

Quantitative analysis of APE/Ref-1-immunopositive cells and TUNEL-positive cells revealed a temporal relationship between reduction of APE/Ref-1 expression and increase in DNA fragmentation in the hippocampal CA1 subregion after transient global ischemia (Figure 5B). The rate of APE/Ref-1-immunopositive cells did not decrease 1 day after ischemia. Two days after ischemia, the APE/Ref-1–positive cells began to decrease significantly and continued to decrease at 3 days. The percentage of TUNEL-positive cells did not increase remarkably until 2 days; however, cells were increased significantly 3 days after ischemia.

Discussion

The present study provides evidence that the reduction of the DNA repair enzyme APE/Ref-1 has a role in the apoptotic cell death pathway in delayed neuronal damage after transient global cerebral ischemia. Delayed neuronal cell death and selective vulnerability in the hippocampal CA1 neurons after transient global ischemia have been investigated in great detail. In this study, we observed by DNA electrophoresis a significant amount of DNA fragmentation in the vulnerable hippocampal CA1 subregion after global ischemia (Figure 1) and TUNEL staining (Figures 2G, 2H, 5A, and 5B). In addition, the time course of increasing TUNEL-positive cells clearly showed that DNA fragmentation occurred in a delayed fashion after transient global ischemia (Figure 5B). However, in light of the increasing evidence that astrocytes and oligodendrocytes could undergo apoptosis after brain injury, we cannot exclude the possibility that the observed DNA laddering may have come from these cells. Because we demonstrated by immunohistochemistry with MAP2 morphological evidence that fragmented DNA cells were mostly from the neuronal cell population (Figure 2), neuronal apoptosis most likely contributed to, if not exclusively caused, DNA fragmentation in the current study. These data implicate the mechanism of delayed neuronal death after global ischemia in the apoptotic pathway, as previously described by others. However, it is still unclear whether DNA fragmentation in hippocampal neurons after transient global ischemia is entirely due to the apoptotic pathway. Also, the neuronal nature of the cells that exhibited DNA fragmentation is not clear at present. Further studies using double staining consisting of a neuronal marker (NeuN) and TUNEL are needed to address this issue. Other studies may include the expression and activation of neuronal caspase-3 or the release of cytochrome c. These studies are under way in our laboratory.

However, little is known about the interaction between the DNA repair mechanism and the apoptotic process, except for the data suggesting a link between apoptosis and the cleavage of the DNA repair enzyme PARP. The interleukin-1β-converting enzyme family of caspases are the human homologues of the nematode Caenorhabditis elegans gene Ced-3 and are considered to play a critical role in programmed cell death. One substrate of caspases is PARP, whose proteolytic cleavage results in a dysfunctional PARP, which is unable to contribute to repair or genomic maintenance. Furthermore, the Ca²⁺/Mg²⁺-dependent endonuclease that generates internucleosomal DNA cleavage characteristic of apoptosis is negatively regulated by poly(ADP-riboseylation). Therefore, inactivation of PARP could increase DNA cleavage and contribute to programmed cell death. On the other hand, excessive activation of PARP is believed to be deleterious because it might cause energy depletion and ultimate cell death. In fact, most recent studies clearly show the reduction of the infarct volume in PARP knockout mice after focal cerebral ischemia. However, the relationship of the DNA repair mechanism to apoptosis in vivo is still unclear because PARP knockout studies involving ischemia have not excluded the role of PARP in apoptotic cell death.
Furthermore, it has been reported that PARP was cleaved in the hippocampus following global ischemia and that the caspase-3 inhibitor reduced neuronal damage and DNA fragmentation (TUNEL-positive cells) in hippocampal CA1 neurons following global ischemia in rats. These results might suggest that different cell death mechanisms involving PARP may exist between focal and global ischemias.

In the case of another major DNA repair enzyme, APE/Ref-1, Robertson et al. reported on the relationship between APE/Ref-1 and apoptosis using the myeloid leukemia cell line HL-60. Inducing apoptosis in HL-60 resulted in down-regulation of APE expression at both the RNA and protein levels. Double labeling using APE/Ref-1 immunohistochemistry and TUNEL staining demonstrated that the cells undergoing apoptosis lost APE/Ref-1 expression. Using rats, Gillardon et al. have shown in vivo that APE/Ref-1 mRNA increased, but the APE/Ref-1 protein decreased in vulnerable hippocampal CA1 neurons following global ischemia induced by cardiac arrest. In the present study, we also clearly showed the decrease of APE/Ref-1 expression in hippocampal CA1 neurons following transient global ischemia (Figure 4). In Western blot analysis, the expression of APE/Ref-1 was decreased after ischemia, whereas the expression of β-tubulin was without effect. These data suggest that reduction of APE/Ref-1 was not caused by nonselective degradation of proteins after ischemia (Figure 3). Moreover, double labeling with APE/Ref-1 and TUNEL staining revealed that all TUNEL-labeled CA1 pyramidal neurons lost APE/Ref-1 immunoreactivity (Figure 5A). In addition, the quantitative analysis of TUNEL-positive and APE/Ref-1–immunopositive cells clarified that the decrease of APE/Ref-1–immunopositive cells preceded the increase of TUNEL-positive cells (Figure 5B). These data suggest that the loss of APE/Ref-1 and the failure of the DNA repair mechanism might contribute to DNA fragmentation after transient global cerebral ischemia in rats. To define this phenomenon, future studies that use transgenic mutant mice with APE/Ref-1 overexpression, deficiency, or selective inhibition of APE/Ref-1 with antisense nucleotides are needed. A decrease in protein synthesis after global ischemia is well known. Furthermore,
an increase in mRNA expression and a decrease in APE/Ref-1 protein levels after transient forebrain ischemia in rats have been reported.\textsuperscript{18} On the other hand, Walton et al\textsuperscript{12} showed the elevation of c-Jun expression at the same time as the decrease in APE/Ref-1 expression after hypoxic-ischemic insult in neonatal rat brains, suggesting the possibility that the loss of APE/Ref-1 protein expression is not due to a nonspecific decrease in protein synthesis. Also, these cells were not fully compromised and retained at least metabolic activity. These results support the hypothesis that the reduction of APE/Ref-1 is involved in the active process of programmed cell death.

The mechanism causing the reduction of APE/Ref-1 immunoreactivity after ischemia/reperfusion is unknown. Because it is well known that reperfusion increases mitochondrial production of superoxide radicals,\textsuperscript{33} the reduced expression of APE/Ref-1 may be due to oxidative damage. APE/Ref-1 has been described as playing a central role in BER by providing a 3'-OH primer for repair synthesis of DNA after all types of oxidative damage.\textsuperscript{34} APE/Ref-1 was induced at both the mRNA and protein levels by exposure to sublethal levels of ROS.\textsuperscript{35} Our recent studies showed that ROS, superoxide in particular, were prominently produced in the hippocampal CA1 subregion 1 day after transient global ischemia.\textsuperscript{12,13} The production of superoxide was restricted in the CA1 subregion, which coincided with the area of APE/Ref-1 decrease. Furthermore, the overexpression of the antioxidant enzyme SOD1 in transgenic rats reduced not only neuronal damage but also DNA fragmentation after transient global ischemia/reperfusion. These data suggest that the ROS might have an important role in the reduction of APE/Ref-1. Therefore, the delayed decrease of APE/Ref-1 might have an important role in the reduction of APE/Ref-1. We are currently studying whether APE/Ref-1 expression is affected by SOD1 overexpression in transgenic rats.

In addition to its important role in the DNA repair mechanism, APE/Ref-1 is drawing particular attention because of its critical role in redox regulation of DNA-binding activity of the activator protein-1 family members, such as Fos and Jun transcription factors,\textsuperscript{36–39} which are also considered to be associated with the pathogenesis of cerebral ischemia.\textsuperscript{40–42} However, recent in vivo studies showed a lack of correlation between APE/Ref-1 protein levels and the expression of inducible transcription factors c-fos and c-jun, suggesting that APE/Ref-1 protein is more likely to be involved in the repair of spontaneous DNA damage than posttranslational modifications.\textsuperscript{43,44} The relationship of APE/Ref-1 reduction to other redox regulating proteins, such as thioredoxin, and to transcription factor activity after ischemia, should also be determined in a future study.

Delayed neuronal death has been explained by many hypotheses, including glutamate toxicity,\textsuperscript{45} protein synthesis inhibition,\textsuperscript{46,47} neurotrophic factor,\textsuperscript{48,49} mitochondrial dysfunction,\textsuperscript{50} and oxygen radicals.\textsuperscript{51–55} Our latest study revealed that the APE/Ref-1 protein began to reduce within 5 minutes after reperfusion following 60 minutes of focal ischemia.\textsuperscript{56} However, superoxide was maximized at 1 day, and APE/Ref-1 expression decreased 2 days after ischemia. Therefore, we propose that the delayed decrease of APE/Ref-1 may be attributed directly to delayed neuronal death after global ischemia. However, the somewhat delayed decrease of APE/Ref-1 protein expression may offer a window of opportunity for therapeutic intervention for neuronal death through apoptosis after transient global ischemia, such as in cardiac arrest, in clinical practice.

In conclusion, we have shown that APE/Ref-1 decreased after transient global ischemia and that this reduction preceded the occurrence of ischemic apoptosis and cell death. Furthermore, we elucidated the temporal and anatomic relationship between the APE/Ref-1 decrease and DNA fragmentation after ischemia/reperfusion. Our results suggest the possibility that the decrease of APE/Ref-1 and the failure of the DNA repair mechanism may contribute to apoptosis after transient global cerebral ischemia.

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APE/Ref-1 Reduction After Global Ischemia


Editorial Comment

In the accompanying article, Kawase and colleagues provide evidence that after transient global ischemia in rat, the expression of the DNA repair enzyme APE/Ref-1 is downregulated in vulnerable CA1 hippocampal cells. Such downregulation precedes internucleosomal DNA fragmentation in these cells, one of the hallmarks of apoptosis. The findings, in concert with evidence from previous studies in culture, raise the possibility that downregulation of APE/Ref-1 expression is one of the factors initiating apoptosis following cerebral ischemia.

Little is known about the mechanisms of DNA damage and repair induced by cerebral ischemia. Attacks to the DNA by ischemia-induced reactive oxygen species, including nitric oxide and its oxidation products, produce single- and double-stranded breaks into the deoxyribose backbone of the molecule as well as damage to individual bases.1,2 DNA damage is counteracted by various pathways of DNA repair. APE/Ref-1 is involved in the repair of damaged bases. The careful and well-controlled study by Kawase et al demonstrates that APE/Ref-1 expression is reduced in vulnerable regions of the ischemic hippocampus. The associated reduction in APE/Ref-1 activity is likely to leave AP sites unrepaird which, in turn, may block DNA replication and induce mutagenicity.3 The observation that DNA fragmentation develops in cells in which APE/Ref-1 is reduced raises the possibility that cells with unrepaired AP sites undergo programmed cell death because they are no longer viable. Further experimental evidence is needed to demonstrate whether this possibility is true. Irrespective of the link to apoptosis, however, Kawase et al provide new and important information that will serve as a starting point for future investigations in this area of research.

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References
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