Metalloporphyrin-Based Superoxide Dismutase Mimic Attenuates the Nuclear Translocation of Apoptosis-Inducing Factor and the Subsequent DNA Fragmentation After Permanent Focal Cerebral Ischemia in Mice

Byung I. Lee, MD; Pak H. Chan, PhD; Gyung W. Kim, MD, PhD

Background and Purpose—Recently, apoptosis-inducing factor (AIF), a mitochondrial proapoptotic protein, and its nuclear translocation have been reported in caspase-independent neuronal apoptosis. In this study, we investigated the contribution of reactive oxygen species (ROS) to the nuclear translocation of AIF and the subsequent DNA fragmentation after permanent focal cerebral ischemia (pFCI) using manganese tetrakis (4-benzoic acid) porphyrin (MnTBAP), which mimics mitochondrial superoxide dismutase.

Method—Adult male ICR mice were subjected to pFCI by intraluminal suture blockade of the middle cerebral artery. Immunohistochemistry and Western blot analysis were performed. Large-scale DNA fragmentation was evaluated by pulse field gel electrophoresis, and apoptotic cell death was quantified. MnTBAP was injected into the ventricle to determine whether the removal of ROS contributes to AIF translocation and the subsequent DNA fragmentation.

Results—Western blot analysis showed that the nuclear translocation of AIF occurred as early as 2 hours after pFCI. AIF translocation was not blocked by a pan-caspase inhibitor. MnTBAP-treated mice had attenuated AIF translocation and blocked large-scale DNA fragmentation. Caspase-3 activity was similarly inhibited between the pan-caspase inhibitor- and MnTBAP-treated mice, but the amount of apoptosis-associated DNA fragmentation in the MnTBAP-treated mice was less than in the pan-caspase inhibitor–treated mice ($P<0.001$).

Conclusion—These results suggest that the MnTBAP, a mitochondrial O$_2^-$ scavenger, may attenuate the caspase-independent nuclear translocation of AIF after pFCI and subsequent apoptosis-associated DNA fragmentation. *(Stroke. 2005;36:2712-2717.)*

**Key Words:** antioxidants, apoptosis, cerebral ischemia, focal, mice

Oxidative stress has been known to be involved in the pathogenesis of cerebral ischemia by direct oxidative cellular damage such as DNA damage or oxidative activation of signaling pathways involving mitochondria and transcriptional factors. Among antioxidant enzymes, manganese superoxide dismutase, which scavenges O$_2^-$ in mitochondria, prevents release of mitochondrial proapoptotic molecule, cytochrome c, and subsequent apoptosis-associated cell death after permanent focal cerebral ischemia (pFCI).1–3 Evidence suggests that apoptosis-inducing factor (AIF), a novel proapoptotic molecule, is a key initiator of alternative caspase-independent pathways involved in apoptotic DNA degradation and apoptotic cell death.4–8 AIF is a mitochondria-localized apoptotic effector protein that induces peripheral chromatin condensation and large-scale ($\approx$50 kbp) fragmentation of chromosomal DNA.4,8 In addition, after treatment with excitotoxin, the neurons treated with neutralizing antibody to AIF or AIF knockdown Haliquin mice showed significantly reduced neuronal injury compared with the controls, suggesting that AIF translocation has a crucial role in the excitotoxic neuronal death.9,10 Recent reports show that the nuclear translocation of AIF could be involved in the mechanism of caspase-independent neuronal cell death after transient focal cerebral ischemia (tFCI) in rats11,12 or mice13 after transient global cerebral ischemia in rats14 and after hypoxia–ischemia in neonatal rat brains.15 In this study, using manganese tetrakis (4-benzoic acid) porphyrin (MnTBAP), a cell-permeable mitochondrial O$_2^-$ scavenger,16 we investigated the relationship between oxidative stress and the nuclear translocation of AIF after pFCI and subsequent apoptosis-associated DNA fragmentation.

Materials and Methods

Focal Cerebral Ischemia

Adult male ICR mice (3 months; 35 to 40 g; Daehan Biolink Co; Chumbuk, South Korea) were subjected to pFCI by intraluminal...
middle cerebral artery (MCA) blockade with an 11.0-mm 5-0 surgical monofilament nylon suture, as described previously.3,17,18 At the end of surgery, the nylon suture was tightly fixed at the final position with a silk suture. Cannulation of a femoral artery allowed the monitoring of blood pressure and arterial blood gas with a gas analyzer (Roche Diagnostics). All procedures were approved by the animal care committee at Yonsei University Medical College.

We injected 2 µL of MnTBAP16 (concentration 50 µg/µL in distilled water; Biomol International) or vehicle (2 µL of distilled water) into the ventricle. To examine the effect of the caspase on the translocation of AIF, we injected 2 µL of N-benzylxoycarbonyl-Val-Val-Asp-Fluoromethyl ketone (z-VAD.fmk), a nonselective caspase inhibitor (125 ng in 0.25% dimethylsulfoxide in PBS [first dissolved in dimethylsulfoxide and then suspended in PBS]) and the vehicle (0.25% dimethylsulfoxide in PBS) intracerebroventricularly 30 minutes after ischemia.19

Caspase-3 Activity Assay
To quantify the caspase-3 activation, we used a commercially prepared enzyme immunoassay (Chemicon International).19 The cytosolic samples were prepared as described for the Western blot method (n=4 each). Cytosolic samples (20 µg of protein) were used to determine the cleaved bioluminescent substrate specifically by active caspase-3 and used for the ELISA, following manufacturer protocol.

Pulsed Field Gel Electrophoresis
For the control, normal or contralateral brain tissues were taken after the brain was cut coronally. The procedure of pulse field gel electrophoresis (PFGE) was followed with the method reported previously.20 Chromosomal DNA samples were prepared in agarose plugs using a CHEF Mammalian Genomic DNA Plug Kit (Bio-Rad). PFGE was performed using a field inverse gel electrophoretic system (Bio-Rad), and the gel was stained with ethidium bromide and visualized under UV light.

Detection of Superoxide Radicals After Ischemia
To confirm the occurrence of O2- after pFCI, in situ detection of oxidized hydroethidion (HET) was performed at 2 hours after ischemia after the previous method.21 A total of 200 µL of HET (stock solution 100 mg/mL in dimethyl sulfoxide; Molecular Probes) was administrated intravenously 30 minutes before scarifying, and prepared samples were observed with a microscope and computerized digital camera system under fluorescent light (excitation=510 to 550 nm and emission >580 nm; BX51; Olympus). Intensity (optical density [OD]) in high-magnification field (×400) and expression patterns of the oxidized HET were analyzed with a computerized analysis system and program (MetaMorpho Imaging; Molecular Devices).

Subfractionization of Cellular Proteins and Western Blot Analysis
To separate the mitochondrial, cytosolic, and nuclear fractions of the proteins, each sample was homogenized briefly in lysis buffer and then centrifuged at 750g for 10 minutes at 4°C. Samples were obtained from the MCA territory brain tissue on the ischemic regions and from the nonischemic controls. Western blot analysis was performed as described previously with modifications.14,15,20 Primary antibodies were used at a 1:200 dilution of goat polyclonal antibodies (Santa Cruz Biotechnology). After incubating with the antibodies, the proteins on the membrane were reacted with horseradish peroxidase–conjugated anti-rabbit IgG with the ECL (enhanced luminescence) plus kit (Amersham International). The relative amounts of the proteins were quantified by densitometric scanning using the Image Analyzer LAS-1000 plus (Fuji Film Co).

Cell Death Assay
To quantify apoptosis-related DNA fragmentation, we used a commercial enzyme immunoassay to determine cytoplasmic histone-associated DNA fragments (Roche Diagnostics), which detect apoptotic oligo-DNA fragmentation.19 Fresh brain tissue was cut into pieces at 1, 2, 4, 8, and 24 hours after ischemia and subfractionized in lysis buffer. A cytosolic volume containing 20 µg of protein was used for the ELISA following manufacturer protocol.

Immunofluorescent Staining for AIF Protein
The fixed sections were incubated with the blocking solution as described previously19 and incubated with primary antibodies, goat polyclonal antibodies for AIF, at a dilution of 1:100 (Santa Cruz Biotechnology). After washing, the sections were incubated with Cy3-conjugated rabbit anti-goat antibodies (1:200; Jackson ImmunoResearch). For neuron-specific nuclear protein (NeuN) staining, the sections were incubated with mouse monoclonal antibodies for NeuN (1:100; PharMingen) after biotinylolation and a blocking procedure using the manufacturer protocol (DAKO ARK; DAKO). After washing, fluorescent avidin D Cell Sorter grade (50 µg/mL; Vector) was applied. Sections were examined under a LSM510 confocal laser scanning microscope (Carl Zeiss).

Double-Fluorescent Staining for AIF and TUNEL
AIF immunohistochemistry was performed as described above. After washing, sections were incubated with 50 µL of TUNEL reaction mixture (terminal deoxynucleotid transferase and fluorescein-dUTP; Roche) on each sample for 60 minutes at 37°C in a dark chamber, then washed and mounted using Vectashield mounting medium. These sections were observed under a confocal laser scanning microscope (Carl Zeiss).

Quantification and Statistical Analysis
The data are expressed as mean±SD. The statistical comparisons among multiple groups were made by ANOVA, followed by the Fisher protected least significant difference test, whereas comparisons between 2 groups were performed by the unpaired t test (StatView; SAS Institute, Inc). The significance between groups was assigned at P<0.05.

Results
Early Nuclear Translocation of AIF After pFCI
As shown in the Western blot analysis of AIF (Figure 1A), AIF immunoreactivity was evident as a molecular mass of ≈67 kDa in the nuclear fraction in the ischemic brain as early as 1 hour after permanent MCA occlusion, where it was not detected in either the normal or the contralateral brain (lane 1). The amount of nuclear AIF protein was increased in a time-dependent manner after pFCI (OD 1 hour, 6.40±0.36; 2 hours, 9.50±0.41; 4 hours, 11.80±0.43; 24 hours, 23.10±0.84; Figure 1A, nuclear fraction). A significant amount of mitochondrial AIF was detected in the nonischemic brain, and this was decreased at 1, 2, and 4 hours after pFCI but not at 24 hours after pFCI (OD control, 25±0.95; 1 hour, 20.25±1.25; 2 hours, 17.25±0.95; 4 hours, 14.50±1.29; and 24 hours, 31.25±5.67; Figure 1A, mitochondrial fraction).

AIF immunofluorescent staining showed a weakly stained perinuclear punctuate pattern in cytosol, which is typical for mitochondrial localization,18 and these cells were colocalized with NeuN-positive cells in the nonischemic brain (Figure 1B1 to 1B3). As shown in Figure 1B4 to 1B6, AIF immunoreactivity was strongly detected in the nucleus of neuronal cells, with NeuN immunoreactivity at 4 hours after pFCI (Figure 1B4 to 1B6 arrowheads). The data on the nuclear AIF
expression in immunohistochemistry are summarized in the Table.

Reduction of Superoxide-Radical Production and AIF-Nuclear Translocation After Treatment of MnTBAP

The production $O_2^-$ was shown by oxidized HEt signals as red particles in the cytosol after ischemia (Figure 2). At 4 hours after permanent ischemia in vehicle-treated mice (Figure 2A1 to 2A2), the ischemic area showed a significantly increased expression of the oxidized HEt signals compared with the MnTBAP-treated mice (Figure 2A3 to 2A4). The average OD of the oxidized HEt signals from the MnTBAP-treated group was significantly lower than in the vehicle-treated group at 4 hours after permanent FCI (OD vehicle 0.68 ± 0.03; MnTBAP 0.33 ± 0.05; $P<0.001$; unpaired t test). In the Western blot analysis, the mean OD of nuclear AIF protein in MnTBAP-treated mice was significantly lower than in the vehicle-treated mice (OD vehicle 11.92 ± 0.94; MnTBAP 2.60 ± 0.27; $P<0.001$; Figure 2B).

Large-Scale DNA Fragmentation Was Blocked in MnTBAP-Treated Mice After Ischemia

DNA fragments $\approx 50$ kbp in size (large-scale DNA fragmentation), by using PFGE, were detected in the ischemic brain at 8 hours after pFCl and were profoundly increased at 16 and 24 hours after ischemia (Figure 3A). Large-scale DNA fragmentation was not seen in the MnTBAP-treated mice, whereas a significant amount of large-scale DNA fragmen-
Caspase-Independent Nuclear Translocation of AIF and Its Involvement in the Apoptosis-Associated DNA Fragmentation

Western blot analysis revealed that the amount of AIF protein was not significantly different between the vehicle- and z-VAD.fmk–treated mice (OD vehicle 11.62±0.69; z-VAD.fmk 11.16±0.82; P>0.05; Figure 4A). Double labeling with AIF and TUNEL was performed at 24 hours after pFCI (Figure 4B). AIF-positive cells were colocalized with TUNEL-positive cells in the ischemic brain but not always concurrent (Figure 4B3, arrowheads).

Caspase-3 activity was significantly diminished in the z-VAD.fmk– and MnTBAP-treated mice compared with the vehicle-treated mice at 24 hours after ischemia (OD vehicle 445.70±15.10; z-VAD.fmk 276.30±13.30; MnTBAP 287.00±10.00% of nonischemic control brain; P<0.001; Figure 4C, left graph). Caspase activity was not different between the z-VAD.fmk– and MnTBAP-treated mice (P>0.05; Figure 4C, left graph). The amount of apoptotic DNA fragmentation significantly decreased in z-VAD.fmk– and the MnTBAP-treated mice compared with the vehicle-treated mice (OD vehicle 515.00±58.00; z-VAD.fmk 399±31.80; MnTBAP 236.00±49.00% of nonischemic control brain; P<0.001; Figure 4C, right graph). Also, the amount of apoptotic DNA fragmentation in MnTBAP-treated mice was significantly less than in z-VAD.fmk–treated mice (P<0.001; Figure 4C, right graph).

Discussion

The current study provides the first evidence that MnTBAP reduces the caspase-independent nuclear translocation of AIF after pFCI and the subsequent neuronal apoptosis-associated DNA fragmentation. Our findings are as follows. First, mitochondrial AIF was reduced in the ischemic brain at 2 hours after pFCI, and the nuclear fraction showed a corresponding increase of AIF in the ischemic brain after pFCI (Figure 1). Second, a postischemic increase of nuclear AIF was significantly reduced at 4 hours after pFCI in MnTBAP-treated mice (Figure 2B). Third, a significant amount of
large-scale DNA fragmentation, using PFGE, was detected at 24 hours after pFCI in vehicle-treated mice and was blocked in the MnTBAP-treated mice (Figure 3). Finally, nuclear translocation of AIF was not affected by the pan-caspase inhibitor z-VAD.fmk in the ischemic lesion after pFCI (Figure 4).

Our present result, which shows that early nuclear translocation of AIF occurred after pFCI (Figure 1) is consistent with previous reports, which showed that AIF translocated into nucleus at 4 to 8 hours after tFCI in rat. In addition, in our present result, the mitochondrial AIF increased at 24 hours after ischemia (Figure 1). Although the role of the increased AIF is unknown, this result is in agreement with the previous result, which states that the mitochondrial AIF decreased at 8 hours but increased at 24 and 48 hours after tFCI in rat. It has been proposed that AIF may act as a cell-saving factor, such as free radical scavenger, when localized in the mitochondrial intermembrane space under normal condition, whereas under the conditions leading to apoptosis, AIF translocates to the nucleus, resulting in DNA fragmentation. However, it is not clear whether the increased AIF in mitochondria has advantageous effects to the cells. Further thorough studies may be required to elucidate the increased mitochondrial AIF of the present study. This study shows that the MnTBAP significantly attenuated the overproduced O$_2^-$ after ischemia, the nuclear translocation of AIF (Figure 2), and the subsequent large-scale DNA fragmentation (Figure 3), suggesting that O$_2^-$ generation in mitochondria may contribute to the nuclear translocation of AIF and the subsequent DNA fragmentation. These are in agreement with the recent in vitro evidence that nuclear translocation of AIF and the subsequent DNA fragmentation may be induced by peroxynitrite in the neuronal cell culture, and by H$_2$O$_2$ in fibroblast culture, suggesting that reactive oxygen species (ROS) may contribute to AIF translocation. However, the exact mechanism of ROS-related nuclear translocation of AIF is unknown. Further experiment to elucidate the regulation of AIF translocation by ROS is required.

In the present study, a part of AIF-positive cells was colocalized with TUNEL-positive cells at 24 hours after pFCI (Figure 4B). Although it is not clear how AIF facilitates DNA fragmentation, this result is consistent with previous reports that most neurons with nuclear AIF also had evidence of DNA fragmentation (TUNEL-positive) at 24 hours after ischemia, with peroxynitrite in the neuronal cell culture, and by H$_2$O$_2$ in fibroblast culture, suggesting that reactive oxygen species (ROS) may contribute to AIF translocation. However, the exact mechanism of ROS-related nuclear translocation of AIF is unknown. Further experiment to elucidate the regulation of AIF translocation by ROS is required.

In conclusion, we have shown that MnTBAP, an O$_2^-$ scavenger, reduces caspase-independent nuclear translocation of AIF after pFCI and subsequent apoptosis-associated cell death. Therefore, reducing O$_2^-$ using MnTBAP may prevent ischemic neuronal cell demise after pFCI by inhibiting not only the caspase-dependent pathways but also the caspase-independent ones.

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