LABORATORY INVESTIGATION

J Korean Neurosurg Soc 41 : 306-313, 2007

# Temporal Characteristics of Cytosolic Translocation of Mitochondrial Proteins in Permanent Distal Middle Cerebral Artery Occlusion Model of Rats

Byoung Wook Shin, M.D., Jae Hoon Sung, M.D., Jae Taek Hong, M.D., Byung Chul Son, M.D., Sang Won Lee, M.D., Chun Kun Park, M.D.

*Department of Neurosurgery, St. Vincent's Hospital, The Catholic University of Korea, Suwon, Korea*

**Objective :** In permanent distal middle cerebral artery occlusion (pdMCAO) model of rats, the temporal order of subcellular translocation is not fully understood yet. We studied translocation sequence of cytochrome c and apoptosis inducing factor (AIF) after pdMCAO and patterns of expression.

**Methods :** Twenty-one male rats - with ten minutes, 1, 4, 8, 24 and 48 hours of pdMCAO groups - were enrolled. At core and penumbra area of each cerebral cortex, Western blotting of cytochrome c and AIF were performed using cytosolic fractions and then compared with sham specimens. With 48 hours group, the expression of cytochrome c and AIF was examined with immunofluorescent staining.

**Results :** Compared to sham, the cytosolic translocation of cytochrome c significantly increased at all time points ($p<0.05$). As early as 10 min after onset of ischemia, it was increased significantly ($p<0.01$). The cytosolic translocation of AIF showed gradual increase with the passage of time and significantly increased 8 hours after ($p<0.05$). As late as 24 hours and 48 hours after onset of ischemia, there were increased most significantly ($p<0.01$). At penumbra, both proteins failed to show significant increase at all time points. At 48 hours after ischemia, colocalization of cytochrome c and AIF were confirmed.

**Conclusion :** Cytosolic translocation of cytochrome c peaks much earlier than that of AIF in pdMCAO model of rat. Caspase dependent apoptosis activates soon after ischemia and later, it can be reinforced by gradually increasing AIF in ischemic core.

**KEY WORDS :** Apoptosis · Mitochondrial proteins · Cytochrome c · Apoptosis inducing factor · Middle cerebral artery occlusion · Ischemia.

## Introduction

After cerebral ischemic stroke, many neurons and glia die by necrosis, which is characterized by the sudden failure of cellular energy, and swelling and rupture of organelles. On the basis of evidence showing that inhibition of protein synthesis reduced brain injury, MacManus et al[22]. suggested that ischemic cell death was not simply passive process but involved synthesis of new proteins as in programmed cell death, namely apoptosis. Subsequently, several morphological and biochemical features observed in apoptosis have been documented in ischemic brain[18,22]. In ischemic stroke, the fate of cell death is mainly determined by intracellular ATP status. Rapid and drastic depletion of intracellular ATP can cause necrotic cell death, but with relatively preserved intracellular ATP, activation of signaling pathway for programmed cell death can be available[3,9,29].

Once apoptosis is triggered, there are three major pathways by which it may be initiated and controlled-the intrinsic and extrinsic pathway that both lead to caspase-3 activation and the caspase-independent apoptosis inducing factor (AIF) pathway. The intrinsic caspase pathway is activated when cytosolic cytochrome c complexes with Apaf-1 and activates caspase-9. Caspase-9 then cleaves and activates caspase-3[17]. In the extrinsic pathway, caspase-8 is activated through the Fas or tumor necrosis factor-$\alpha$ membrane receptor systems[28]. Activated caspase-8 then cleaves and activates caspase-3. This pathway bypasses the mitochondria. The third pathway by which apoptosis may occur is caspase-independent. A key factor in this pathway is AIF, a novel proapoptotic molecule

• Received : January 24, 2007 • Accepted : March 21, 2007

• Address for reprints : Jae Hoon Sung, M.D., Department of Neurosurgery, St. Vincent's Hospital, The Catholic University of Korea, 93-6 Ji-dong, Paldal-gu, Suwon 442-723, Korea Tel : +82-31-249-8168, Fax : +82-31-245-5208, E-mail : jaehoonsung@gmail.com

that is involved in the final execution of apoptosis[31].

After ischemic insults, markedly increased cytosolic calcium and free radicals are potent injurers of mitochondrial membranes. Various mitochondrial intermembrane proteins, including cytochrome c and AIF, may leak and this phenomenon was named as translocation[39]. In numerous experiments using different in vitro or in vivo conditions, animal species and types of ischemic conditions, the activation patterns of these mitochondrial apoptotic proteins showed some controversies[2,7,10,12,15,26,32,34,38,39]. It is well known that active involvement of mitochondrial apoptotic pathway in focal middle cerebral artery occlusion (MCAO) model of rats. But most of studies omit very early time point of observation and temporal sequence of activation of these proteins. The purpose of this study is to compare the temporal activation sequence and patterns of two representative mitochondrial apoptotic proteins, namely cytochrome c and AIF, with special interest in very early points after focal permanent ischemic model of rats. In addition, the possibility of co-activation of these proteins in delayed ischemia was also investigated.

## Materials and Methods

### Permanent distal middle cerebral artery occlusion (pdMCAO) model

Focal cerebral ischemia was generated as described[37]. Twenty-one (n=3, each group) male Sprague-Dawley rats (250-300 g) were used. Anesthesia was induced by 5% isoflurane and maintained with 1.5-2.5% isoflurane during surgery. The femoral artery was exposed and a polyethylene tube (OD 0.9 mm) was inserted into it. The blood pressure, $PaO_2$, $PaCO_2$, pH and hematocrit were checked serially. Core body temperatures were monitored with a rectal probe and maintained at 37°C. Small vertical incision was made on anterior midline of neck and both common carotid arteries (CCAs) were isolated. After making of small vertical incision on preauricular area and dissection of temporal muscle, small craniectomy was made at the junction of zygomatic arch and squamous bone. Using small aneurysm clips, both CCAs were clamped and exposed distal MCA was cauterized and cut above the rhinal fissure area (Fig. 1). The aneurysm clips on both CCAs were released 1 hour after. In sham surgery control, only craniectomy was made and closed. Using TTC (triphenyltetrazolium chloride) staining, consistency of animal model was confirmed (Fig. 2).

**Fig. 1.** Intraoperative photograph of permanent distal middle cerebral artery occlusion (pdMCAO) of rat. The temporal muscle is cut and retracted with self-retaining retractor. Temporal craniectomy and dura opening reveal bright red-colored, Y shaped MCA (arrow) and dark purple-colored, serpentine middle cerebral vein (arrow head). The most proximal portion of MCA is coagulated and then cut.

### Brain sample preparations

A separate set of animal brains subjected to similar experimental conditions was harvested 10 minutes, 1, 4, 8, 24, and 48 hours after the onset of ischemia. Animals were perfused transcranially with normal saline. The brains were removed and tissue corresponding to the ischemic core and penumbra was dissected for Western blots. First, based on anterior coordinates, 4-8 mm portion was cut and harvested. Second, from the midline, 2 mm sagittal portion was cut and discarded. Third, the cortical infarct portion was cut and bisected to harvest core and penumbra[11,37] (Fig. 3). Prepared sham operation animals were treated with same manner.

### Cytosolic subcellular fractionation

Rat brains were homogenized in 7 volumes of homogenization buffer (20 mM TrisHCl (pH 7.5), 2 mM EDTA, 10 mM EGTA, 250 mM Sucrose plus 0.7% protease and phosphatase inhibitor cocktails (Sigma) and were centrifuged at 100,000 × g for 30 min at 4°C. The supernatants were collected as the cytosolic fraction[4].

### Western blots

Western blots were performed as described[37]. Protein concentration in each cytosolic fraction was determined using Bradford protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each brain sample was mixed with 5 × SDS buffer, boiled for 5 min, and subjected to SDS-PAGE for 1 hour. Protein bands were transferred from the gel to a PVDF membrane (Hybond-P, Amersham Biosciences, Arlington Heights, IL, USA) for 1 hour. Membranes were blocked with 5% milk in PBS containing 0.1% Tween-20 and then probed for cytochrome c (1:1000, rabbit polyclonal, Cell Signal Technology, Danvers, MA, USA) or AIF (1:2000, goat polyclonal, Santa Cruz Biotechnologies, Santa Cruz, CA, USA)

**Fig. 2.** Triphenyltetrazolium chloride(TTC)-stained brains 48 hours after the onset of pdMCAO. White areas indicate regions of infarction. Note the cortically confined infarction with sharp demarcation.

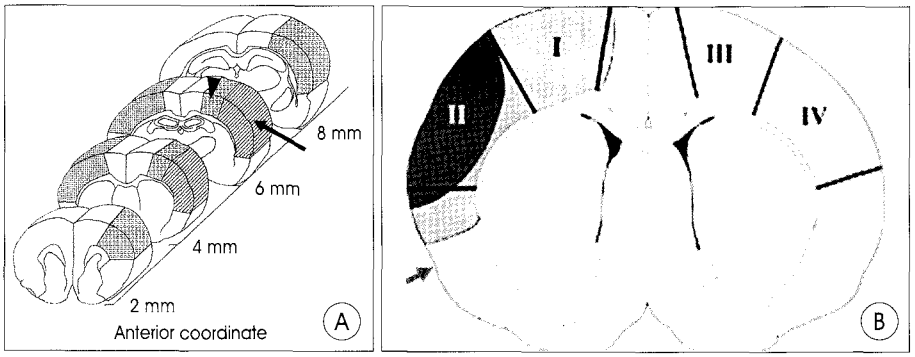


**Fig. 3.** A : Schematic diagram shows delineated infarct margin after pdMCAO. Suggestive core (arrow) and penumbra (arrow head) zones are indicated. B : Schematic diagram shows coronal cut of pdMCAO rat brain. The point of MCA coagulation is indicated (arrow). Area I and II depict ipsilateral ischemic penumbra and core, respectively. Area III and IV depict contralateral nonischemic brain.

by incubating in each primary antibody for 1 hour followed by a horseradish peroxidase-conjugated secondary anti-rabbit or anti-goat IgG antibody (1:2000, Cell Signaling Technology) for 1 hour at room temperature. Protein bands were detected using an enhanced chemiluminescence system (ECL Western blotting detection reagent, Amersham Biosciences) and exposed to BioMax film (Kodak, Rochester, NY, USA). Films were scanned with a photoscanner and analyzed using Image J (US National Institute of Health, Bethesda, MD, USA). To confirm even loading of proteins, membranes were stripped and probed for $\beta$-actin (AC-15, 1:40000, mouse monoclonal, Sigma). Differences in protein bands from Western blots were analyzed using one-way ANOVA followed by Tukey's post hoc test. All tests were considered statistically significant at $p$-values $<0.05$. Data are presented as means $\pm$ S.E.M.

### Histochemistry and Laser confocal microscopy

Double-label fluorescence confocal microscopy was utilized on brain sections from animals subjected to pdMCAO. Animals were killed 48 hours later with an overdose of halothane, and brain sections were prepared and analyzed as previously described[35]. Briefly, rats were perfused transcardially with normal saline followed with 3% paraformaldehyde (PFA). Brains were post-fixed with 3% PFA, 20% sucrose for 24 hours. Thirty-micrometer sections were cut onto glass slides in the coronal plane using a cryostat. They were washed in PBS for $3 \times 10$ minutes and then blocked in PBS containing 5% donkey serum (Sigma, St. Louis, MO) and 0.3% triton X-100 for 2 hours at room temperature. Two primary antibodies were mixed in the blocking solution and applied onto the slides, and incubated at 4°C overnight. Negative controls, in which the primary antibodies were omitted, were run in parallel. For cytochrome c and AIF, mouse anti-cytochrome c antibody (1:200; Pharmingen) and goat anti-AIF antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) were used. After washed with PBS, the sections were incubated in the mixed secondary antibodies for 2 hours at room temperature. For detection of cytochrome c and AIF, Cy-3 conjugated donkey anti-rabbit IgG (1:200; Jackson Immuno Research) and biotinylated anti-goat IgG (1:100; Vector laboratories, Burlingame, CA) were used. For AIF staining, after the secondary antibody incubation, FITC-conjugated streptavidin (1:100; Molecular Probes) was added and incubated for 1 hour at room temperature. After washing in PBS, all the slices were stained by 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) for 2 minutes and mounted and examined under the a LSM530 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY).

## Results

### Permanent distal middle cerebral artery occlusion (pdMCAO) model

After temporal craniectomy and dural opening, distal middle cerebral artery and vein were clearly visible. Middle cerebral artery was coagulated and cut (Fig. 1). TTC staining confirmed uniform and reproducible infarct shadow and margin (Fig. 2).

### Cytosolic translocation of cytochrome c occurred very early time of ischemia

Increased cytochrome c release was observed in pdMCAO model of rats. Western blots indicated that at the core of ischemia, cytosolic cytochrome c increased as early as 10 min after permanent ischemia, and continuously and significantly increased until 48 hours after ($p<0.05$). It was increased most significantly at 10 min, soon after ischemia ($p<0.01$). But at the penumbra, cytochrome c did not show these

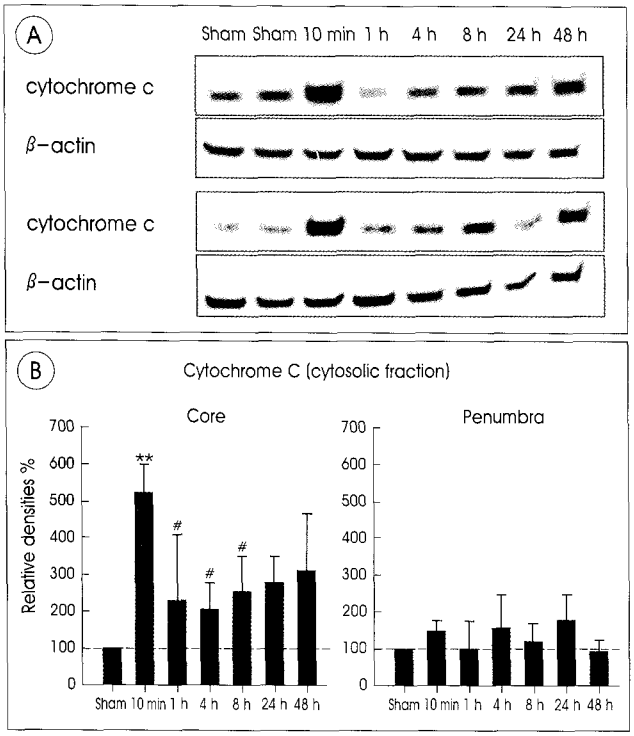


**Fig. 4.** A : Western blots of cytosolic subcellular fractions isolated from ischemic core using cytochrome c antibody after pdMCAO. The most dense cytochrome c band is noted at 10min after onset of ischemia. The band density is gradually increased over time. B : Optical densities of Western blots show significantly increased densities at all time points compared to sham condition ($p<0.05$) and at 10min, it is increased most significantly (**, $p<0.01$). Compared to 10min, they are significantly decreased at 1, 4 and 8hours after onset of ischemia (#, $p<0.05$). At the penumbra, cytochrome c do not show these characteristic and significant translocation patterns compared to sham operation. One-way ANOVA followed by Tukey's post hoc test. Data are presented as means±S.E.M.

characteristic and significant translocation patterns compared with sham operation rats (Fig. 4).

### Cytosolic translocation of AIF occurred gradually after ischemia

AIF also translocated into cytosol after permanent ischemia with quite different manner. Western blots indicated that at the core of ischemia, cytosolic AIF increased gradually after permanent ischemia. The relative densities of each time points showed that peak increase was noted at 48 hours, relatively late time of ischemia. Compared to sham operated rats, significant increase were noted at 8, 24 and 48 hours after ischemia and they showed gradual increase patterns ($p<0.05$). But at the penumbra, AIF did not show these characteristic and significant translocation patterns compared to sham operation rats (Fig. 5).

### Cytosolic translocation of cytochrome c and AIF occurred simultaneously in cells of ischemia

Confocal immunostaining confirmed that cytosolic

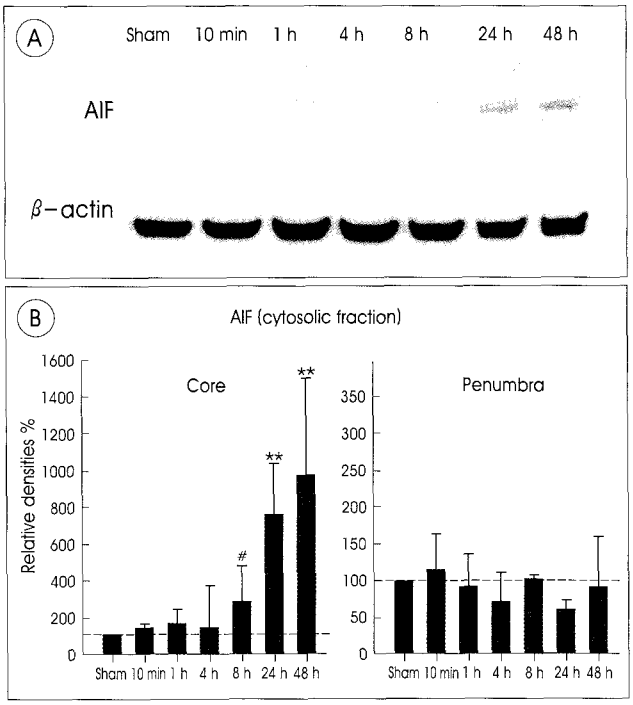


**Fig. 5.** A : Western blots of cytosolic subcellular fractions isolated from ischemic core using AIF antibody after pdMCAO. The dense AIF bands are noted after 24 hours after onset of ischemia. The bands densities are gradually increased over time. B : Optical densities of Western blots show significantly increased densities after 8 hours compared to sham condition (#, $p<0.05$) and at 24 and 48 hours, they are increased more significantly (**, $p<0.01$). At the penumbra, AIF do not show these characteristic and significant translocation patterns compared to sham operation. One-way ANOVA followed by Tukey's post hoc test. Data are presented as means±S.E.M.

translocation of cytochrome c and nuclear and cytoplasmic translocation of AIF in cells of 48 hours after ischemia. Red colored Cy-3 positive cytochrome c immunostaining was noted at the cytoplasm with punctuate manner. Green colored FITC positive AIF immunostaining was noted at the nucleus and cytoplasm of the ischemic apoptotic cell. After merging, they colocalized at a single cell (Fig. 6).

## Discussion

In apoptotic neuronal death of stroke, similar to other apoptosis models, mitochondria play an important role. Mitochondria respond to multiple death stimuli including stroke in which proapoptotic Bcl2 family proteins such as Bax/Bak induce mitochondrial membrane permeabilization and cause the release of apoptotic molecules[25]. The most notorious apoptotic factor released from permeabilized mitochondria is the respiratory component cytochrome c[16]. Cytochrome c is a ubiquitous, heme-containing protein that normally resides in a space between the inner and outer mitochondrial membranes[25]. The polypeptide can be synthesized as an apoprotein in the cytoplasm and is then

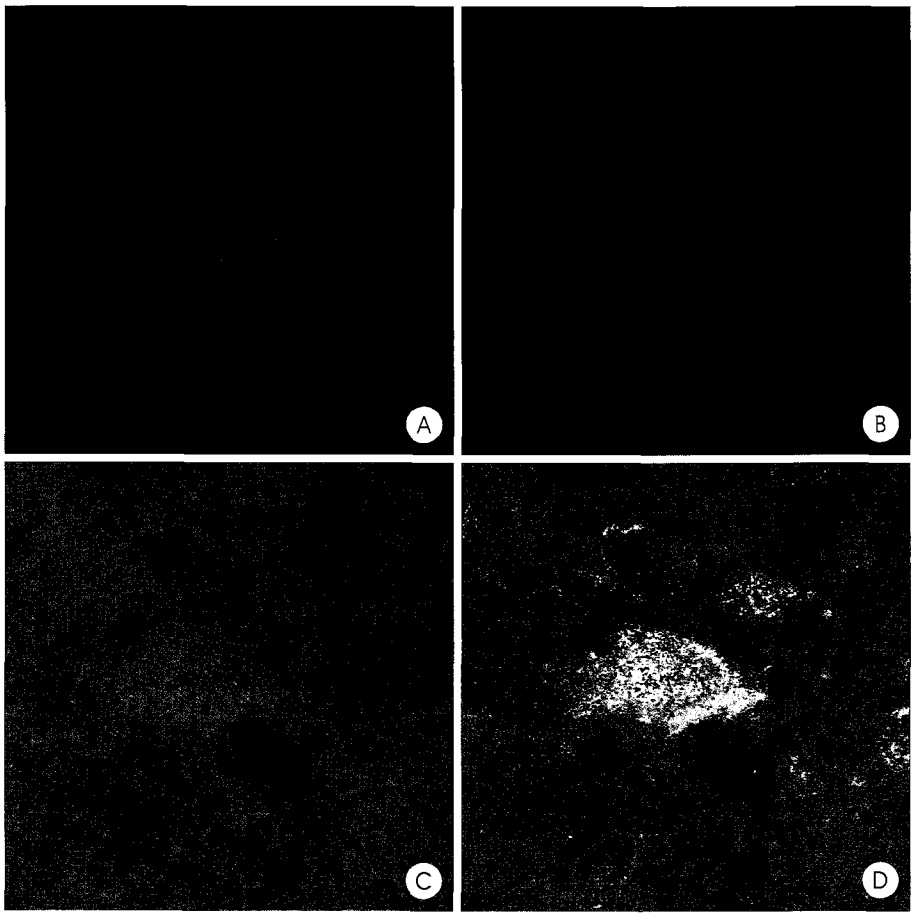


**Fig. 6.** Triple immunofluorescent staining of 48 hours after pdMCAO specimens. A : Cy-3 positive, red colored cytochrome c is localized at cytoplasm with patch densities. B : DAPI positive, blue colored nuclei are visible. Note central ill defined destructed nuclear shadow. C : FITC positive, green colored AIF is localized at cytoplasm and nuclei. D : Merged image definitely shows colocalization of cytoplasmic cytochrome c (red), nuclear and cytoplasmic AIF (green) and the nuclear dye DAPI (blue) within single cortical cells at 48 hours after onset of ischemia. In this model, cytochrome c and AIF can activate simultaneously and execute apoptotic cell death. Bar indicates 10㎛.

transported into the mitochondria. In the intermembrane space, the heme is covalently attached to cytochrome c by a heme lyase, and folding of the polypeptide into its native conformation occurs[24]. In live cells, native cytochrome c is found in the intermembrane space and has function of electron transport chain, leading to the reduction of oxygen to water[25]. In 1996, another role for cytochrome c as an apoptogenic agent was reported[20]. Key to this function was so-called translocation of native cytochrome c from mitochondria to the cytoplasm[14]. The cytosolic cytochrome c may form the apoptosome with procaspase 9 and apaf-1, and cleave procaspase-9 further causing subsequent caspase-3 activity[36,41]. In caspase dependent apoptosis pathway, cytosolic translocation and activation of cytochrome c is clearly upstream of caspase activity and should precede caspase activity. Activated caspases can stimulate cytochrome c release with feedback manner. Indeed, prior works have shown that recombinant caspases cause cytochrome c[23,33] and AIF release in purified mitochondria[23]. Furthermore, caspase inhibition blocks cytochrome c release in purified mitochondria[33].

In cerebral ischemia models, the role and patterns of released or translocated cytochrome c is still controversial and condition specific[10,12,15,26,34]. A study showed no definite evidence of cytochrome c release or caspase activity after permanent focal ischemia[12], but another report revealed no caspase activity despite of cytochrome c release after transient focal ischemia[12,34]. Using global ischemia model, Zhao et al.[39] reported a clear biphasic pattern of cytochrome c release and increased caspase activity just before the second phase of cytochrome c release. In this global ischemia model, an increase in cytochrome c was detected 5 hours after ischemia, followed by a decrease at 12 and 24 hours. A second peak of cytochrome c release was observed at 48 hours. Using apoptosis model of human IM-9 multiple myeloma cells caused by gamma-irradiation and etoposide, two distinct stages of cytochrome c release were also observed[6]. Despite of different situational setup, these biphasic peaks showed common findings; the first peaks were minor degree and second peaks were predominant and main executor of apoptosis. Our experiment of pdMCAO model showed characteristic findings. First, the activation of cytochrome c occurred very early, just 10 minutes after pdMCAO. Most of other studies omit such an early time setup, so this early and massive cytoplasmic translocation of cytochrome c has been ignored until now. Among various mitochondrial apoptotic proteins, cytochrome c may be one of the major participants of apoptosis in this model. Second, after the early surge, the increased cytosolic cytochrome c was continuously and significantly maintained until 48 hours after ischemia, which meant active participation of mitochondrial intrinsic apoptotic cascade including cytochrome c plays an important role in this ischemia model. Considering possible bidirectional feedback stimulation characteristics between cytochrome c and caspase, initial early cytosolic cytochrome c surge can subsequently activate

caspase cascade. Activated caspase may stimulate cytochrome c translocation again, which mean apoptotic cell death is not merely succeeding reaction of necrotic neuronal cell death. It also remains possible that the release of cytochrome c triggers much of the apoptotic machinery and contributes to the death of other cells even though the conditions do not allow the development of full apoptotic morphology. Taken altogether, these controversies, including our results, may be derived from diversities of animal species, model of ischemia, type of perfusion arrest, and fixation and staining methods used. The activation and translocation patterns of cytochrome c may have apoptotic condition specific characteristics.

Apoptosis-inducing factor (AIF) is a mitochondrion-localized flavoprotein with NADH activity that is encoded by a nuclear gene[19,27,32]. Initially, it has been suggested that AIF functions upstream and independent of caspase activation[32]. AIF believed to mediate caspase-independent death because inhibition of caspase activation or caspase activity itself does not abolish the proapoptotic action of AIF[21,31,32]. But, more extensive researches revealed another close relationship between AIF and caspase[2,7]. These findings led to the proposal that apoptosis would follow a general scheme in which different primary stimuli including selective cytochrome c release from mitochondria would stimulate the activation of caspases, which then would trigger the release of AIF from mitochondria[1]. AIF has been shown to translocated from mitochondria to the cytosol as well as nucleus when apoptosis is induced[8,32]. In cytosol, it can generate reactive oxygen species (ROS). The diffuse cytosolic translocation of AIF, which was confirmed by Western blot and confocal microscopy, may reflect a necrotic, passive AIF release in the ischemic core. In nucleus, it can bind DNA and activates endonuclease for nuclear shrinkage without caspase activation[32].

Temporal order of translocation in mitochondrial cytochrome c and AIF also has species and condition specific characteristics. Nuclear AIF translocation was not detected until 24 hours after transient global ischemia[5]. After global ischemia, cytochrome c release occurs as early as 2 hours after global ischemia[30]. In contrast, cytochrome c release occurred after nuclear AIF translocation in neonatal rat brains exposed to hypoxia-ischemia[40]. In rat pdMCAO model, it was reported that AIF translocated into nuclei after cytochrome c was released into the cytosol[38]. In this model, the nuclear and cytoplasmic translocation of AIF occurred with similar temporal characteristic. Both of them occurred after cytosolic cytochrome c translocation and were detected at 8 hours of ischemia and continued until 48 hours[38]. Like previous data, our results also showed gradual increase of cytosolic translocation of AIF at the core of ischemia. Unlikely to cytochrome c, there was no definite early surge at the 10 min point. Our results also showed that, after 48 hours of ischemia, the cytoplasmic cytochrome c, nuclear AIF and cytoplasmic AIF can colocalize at single cell in ischemic core. This means early surged cytoplasmic cytochrome c and corresponding caspase dependent apoptosis would occurr very early and maintain until 48 hours. During 48 hours, AIF induced nuclear apoptotic pathway was activated and at the same time, cytoplasmic AIF turned on necrotic cascade via ROS activation. This suggestion can be supported by immunostaining results. Considering nuclear condensation characteristics of apoptosis, the nucleus of cytochrome c and AIF double stained ischemic cell showed not condensed but dispersed density at the ischemic core. Despite of activation of cytochrome c and AIF, additional necrotic cascade might change nuclear morphology at the ischemic core.

Variations in the duration and intensity of ischemia, energy failure, and protein synthesis inhibition may produce a continuum between the features of necrosis and apoptosis in ischemic models including pdMCAO[13]. At ischemic penumbra, well-known site of apoptosis, we failed to show significant increase of cytochrome c and AIF. It can also be interpretable that acute mitochondrial membrane failure induced passive leakage of these apoptotic proteins at ischemic core. Another possibility is sampling error of penumbra with abundant normal brain tissue. At the ischemic core, our results definitely showed that the biphasic increase of cytochrome c at 10 minutes and 48 hours after onset of ischemia. Considering intracellular decrease pattern of ATP, it is more reasonable that very early translocation of cytochrome c is controlled by apoptosis and lately at 48 hours of ischemia onset, this apoptotic cascade is magnified by additional mitochondrial proteins such as AIF or superimposed by necrosis or shifted into necrosis.

## Conclusion

The cytosolic translocation of cytochrome c occurs much earlier than that of AIF in pdMCAO model of rat. Very early increase of cytosolic cytochrome c translocation shows rapid and active activation of caspase dependent apoptotic pathway in this model. Very early cytochrome c surge may suggest that active, not passive or in the sequel, involvement of mitochondrial apoptotic machinery in this ischemia model. Delayed translocation of AIF can magnify apoptotic cell death as well as can turn on necrotic cascade via cytosolic translocation. The prevention of this pivotal event may be another possible therapeutic strategy, but such an early activation can be a dilemma to solve and overcome.

## References


1. Arnoult D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ : Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. **Embo J 22** : 4385-4399, 2003
2. Arnoult D, Parone P, Martinou JC, Antonsson B, Estaquier J, Ameisen JC : Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli. **J Cell Biol 159** : 923-929, 2002
3. Blomgren K, Zhu C, Hallin U, Hagberg H : Mitochondria and ischemic reperfusion damage in the adult and in the developing brain. **Biochem Biophys Res Commun 304** : 551-559, 2003
4. Bright R, Raval AP, Dembner JM, Perez-Pinzon MA, Steinberg GK, Yenari MA, et al : Protein kinase C delta mediates cerebral reperfusion injury in vivo. **J Neurosci 24** : 6880-6888, 2004
5. Cao G, Clark RS, Pei W, Yin W, Zhang F, Sun FY, et al : Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. **J Cereb Blood Flow Metab 23** : 1137-1150, 2003
6. Chen Q, Gong B, Almasan A : Distinct stages of cytochrome c release from mitochondria : evidence for a feedback amplification loop linking caspase activation to mitochondrial dysfunction in genotoxic stress induced apoptosis. **Cell Death Differ 7** : 227-233, 2000
7. Cregan SP, Fortin A, MacLaurin JG, Callaghan SM, Cecconi F, Yu SW, et al : Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. **J Cell Biol 158** : 507-517, 2002
8. Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, et al : Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. **Faseb J 14** : 729-739, 2000
9. Fiskum G : Mitochondrial participation in ischemic and traumatic neural cell death. **J Neurotrauma 17** : 843-855, 2000
10. Fujimura M, Morita-Fujimura Y, Murakami K, Kawase M, Chan PH : Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. **J Cereb Blood Flow Metab 18** : 1239-1247, 1998
11. Fukuyama N, Takizawa S, Ishida H, Hoshiai K, Shinohara Y, Nakazawa H : Peroxynitrite formation in focal cerebral ischemia-reperfusion in rats occurs predominantly in the peri-infarct region. **J Cereb Blood Flow Metab 18** : 123-129, 1998
12. Gill R, Soriano M, Blomgren K, Hagberg H, Wybrecht R, Miss MT, et al : Role of caspase-3 activation in cerebral ischemia-induced neurodegeneration in adult and neonatal brain. **J Cereb Blood Flow Metab 22** : 420-430, 2002
13. Graham SH, Chen J : Programmed cell death in cerebral ischemia. **J Cereb Blood Flow Metab 21** : 99-109, 2001
14. Green DR, Kroemer G : The pathophysiology of mitochondrial cell death. **Science 305** : 626-629, 2004
15. Hu BR, Liu CL, Ouyang Y, Blomgren K, Siesjo BK : Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. **J Cereb Blood Flow Metab 20** : 1294-1300, 2000
16. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD : The release of cytochrome c from mitochondria : a primary site for Bcl-2 regulation of apoptosis. **Science 275** : 1132-1136, 1997
17. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al : Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. **Cell 91** : 479-489, 1997
18. Li Y, Chopp M, Jiang N, Yao F, Zaloga C : Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. **J Cereb Blood Flow Metab 15** : 389-397, 1995
19. Lipton SA, Bossy-Wetzel E : Dueling activities of AIF in cell death versus survival : DNA binding and redox activity. **Cell 111** : 147-150, 2002
20. Liu X, Kim CN, Yang J, Jemmerson R, Wang X : Induction of apoptotic program in cell-free extracts : requirement for dATP and cytochrome c. **Cell 86** : 147-157, 1996
21. Loeffler M, Daugas E, Susin SA, Zamzami N, Metivier D, Nieminen AL, et al : Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor. **Faseb J 15** : 758-767, 2001
22. MacManus JP, Linnik MD : Gene expression induced by cerebral ischemia : an apoptotic perspective. **J Cereb Blood Flow Metab 17** : 815-832, 1997
23. Marzo I, Susin SA, Petit PX, Ravagnan L, Brenner C, Larochette N, et al : Caspases disrupt mitochondrial membrane barrier function. **FEBS Lett 427** : 198-202, 1998
24. Mayer A, Neupert W, Lill R : Translocation of apocytochrome c across the outer membrane of mitochondria. **J Biol Chem 270** : 12390-12397, 1995
25. Newmeyer DD, Ferguson-Miller S : Mitochondria : releasing power for life and unleashing the machineries of death. **Cell 112** : 481-490, 2003
26. Ouyang YB, Tan Y, Comb M, Liu CL, Martone ME, Siesjo BK, et al : Survival- and death-promoting events after transient cerebral ischemia : phosphorylation of Akt, release of cytochrome C and Activation of caspase-like proteases. **J Cereb Blood Flow Metab 19** : 1126-1135, 1999
27. Penninger JM, Kroemer G : Mitochondria, AIF and caspases-rivaling for cell death execution. **Nat Cell Biol 5** : 97-99, 2003
28. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al : Two CD95 (APO-1/Fas) signaling pathways. **Embo J 17** : 1675-1687, 1998
29. Shin CS, Choi BY, Jung ES, Kim SW, Chang CH, Cho SH : The time evolution of cerebral apoptosis in the permanent middle cerebral artery occlusion model of rats. **J Korean Neurosurg Soc 37** : 54-58, 2005
30. Sugawara T, Fujimura M, Morita-Fujimura Y, Kawase M, Chan PH : Mitochondrial release of cytochrome c corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia. **J Neurosci 19** : RC39, 1999
31. Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, et al : Two distinct pathways leading to nuclear apoptosis. **J Exp Med 192** : 571-580, 2000
32. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al : Molecular characterization of mitochondrial apoptosis-inducing factor. **Nature 397** : 441-446, 1999
33. Xia T, Jiang C, Li L, Wu C, Chen Q, Liu SS : A study on permeability transition pore opening and cytochrome c release from mitochondria, induced by caspase-3 in vitro. **FEBS Lett 510** : 62-66, 2002
34. Yenari MA, Iwayama S, Cheng D, Sun GH, Fujimura M, Morita-Fujimura Y, et al : Mild hypothermia attenuates cytochrome c release but does not alter Bcl-2 expression or caspase activation after experimental stroke. **J Cereb Blood Flow Metab 22** : 29-38, 2002
35. Yenari MA, Minami M, Sun GH, Meier TJ, Kunis DM, McLaughlin JR, et al : Calbindin d28k overexpression protects striatal neurons from transient focal cerebral ischemia. **Stroke 32** : 1028-1035, 2001
36. Yuan J, Horvitz HR : A first insight into the molecular mechanisms of apoptosis. **Cell 116** : S53-56, 51 p following S59, 2004
37. Zhao H, Shimohata T, Wang JQ, Sun G, Schaal DW, Sapolsky RM, et al : Akt contributes to neuroprotection by hypothermia against cerebral ischemia in rats. **J Neurosci 25** : 9794-9806, 2005
38. Zhao H, Yenari MA, Cheng D, Barreto-Chang OL, Sapolsky RM, Steinberg GK : Bcl-2 transfection via herpes simplex virus blocks apoptosis-inducing factor translocation after focal ischemia in the rat. **J Cereb Blood Flow Metab 24** : 681-692, 2004
39. Zhao H, Yenari MA, Cheng D, Sapolsky RM, Steinberg GK : Biphasic cytochrome c release after transient global ischemia and its inhibition by hypothermia. **J Cereb Blood Flow Metab 25** : 1119-1129, 2005
40. Zhu C, Qiu L, Wang X, Hallin U, Cande C, Kroemer G, et al : Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. **J Neurochem 86** : 306-317, 2003
41. Zou H, Li Y, Liu X, Wang X : An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. **J Biol Chem 274** : 11549-11556, 1999


# Commentary

The benefits of this study into understanding of ischemic neuronal insults are significant, but neurosurgeons as well as neuroscientists must participate in debates over the pathophysiologic implications of their findings. Temporal profiles of cytoplasmic translocation of apoptogenic mitochondrial proteins are updated in this study and are also

well correlated with previous findings. Most of all, premature release of cytochromc-c into the cytosol is an important observation that could change the current opinion in the initiation of apoptotic cascade in ischemic insults. However, they failed to show any increase of the proteins in the cytosol of ischemic penumbra. Therefore, their results should be interpreted as an epiphenomenon which is more likely to relate to necrotic release of the proteins from mitochondria in the ischemic core of their permanent MCAO model, until they can show the identical findings at the ischemic penumbra where the amount of ATP for ionic homeostasis is supplied and thus apoptotic cell deaths are likely to occur. Based on the principle of Gibbs-Donnan equilibrium, $Na^+$-$K^+$ pump failure seen in energy-depleted areas (e.g. ischemic core) leads inevitably to osmotic cell swelling and rupture of biologic membrane.

Youn-Kwan Park, M.D., Ph.D.
Department of Neurosurgery, Korea University

## Commentary

The authors use a permanent distal middle cerebral artery occlusion model in the rat to observe the temporal characteristics of cytosolic translocation of mitochondrial proteins, cytochrome c and apoptosis inducing factor (AIF). In cerebral ischemia two executional pathways contribute to apoptosis : the extrinsic pathway which is triggered by the binding of ligand to death receptor and the intrinsic pathway, also known as mitochondrial pathway. Each of those pathways contains both caspase-dependant pathway and caspase-independant pathway. Mitochondria have been considered the most important organelle in the signaling process of apoptosis for its role in the regulation and amplification of apoptotic signals. Cytochrome c is an important factor to activate caspase-independant pathway and AIF is an key factor to activate caspase-independant pathway. In this carefully designed and well organizing animal experimental study the authors document that the cytosolic translocation of cytochrome c significantly increase as early as 10 minutes after onset of permanent ischemia in ischemic core and continuously increase until 48 hours after. They also document cytosolic translocation of AIF shows gradual increase with the passage of permanent ischemic time and significantly increase 8 hours after. They confirm colocalization of cytochrome c and AIF at 48 hours after permanent ischemia. However, at ischemic penumbra, both mitochondrial proteins fail to show significant increase at all time.

The article highlights the activation of cytochrome c occurs very early, just 10 minutes after permanent ischemia. This finding has been ignored in most of other studies. As authors describe, this early surge and continuous and significant increase of cytosolic cytochrome c suggests cytochrome c plays an important role in intrinsic caspase-dependant pathway of apoptosis in this permanent ischemic model. They confirm delayed gradual and diffuse cytosolic translocation of AIF at the core of ischemia in this model and suggest that it may reflect a necrotic, passive AIF release. However, they fail to show significant increase of cytochrome c and AIF at ischemic penumbra which has been a well known site of apoptosis and suggest some possible causes. I think the most plausible cause might be either wrong defining or sampling of penumbra. The authors suggest an important potential therapeutic strategy for future research in the field of permanent cerebral ischemia.

Sae-Moon Oh, M.D.
Department of Neurosurgery, Hallym University