Superoxide During Reperfusion Contributes to Caspase-8 Expression and Apoptosis After Transient Focal Stroke

Yuiko Morita-Fujimura, PhD; Miki Fujimura, MD, PhD; Takashi Yoshimoto, MD, PhD; Pak H. Chan, PhD

Background and Purpose—Reactive oxygen species produced during reperfusion may play a detrimental role in focal cerebral ischemia (FCI). We examined the protein expression of caspase-8, which plays a major role in both Fas-dependent and cytochrome c–dependent apoptotic pathways after FCI with or without reperfusion. Caspase-8 expression after transient FCI was compared between wild-type and transgenic mice that overexpress the cytosolic antioxidant copper/zinc superoxide dismutase (SOD1).

Methods—Adult male CD-1 mice were subjected to 1 hour of FCI and reperfusion or to permanent FCI by intraluminal blockade of the middle cerebral artery. DNA fragmentation was evaluated by genomic DNA gel electrophoresis. Caspase-8 expression was analyzed by Western blot.

Results—Caspase-8 was significantly induced 4 hours after transient FCI and remained at an increased level until 24 hours, whereas it was not modified after permanent FCI. Genomic DNA gel electrophoresis showed DNA laddering in a pattern similar to that seen in apoptosis, with a small amount of background smear 24 hours after transient FCI, whereas 25 hours of permanent FCI resulted in less DNA laddering with a strong background smear. Caspase-8 induction was significantly reduced in SOD1 transgenic mice compared with wild-type mice 4 hours after transient FCI.

Conclusions—The results suggest that increased reactive oxygen species production during reperfusion may contribute to the induction of caspase-8, thereby exacerbating apoptosis after FCI. (Stroke. 2001;32:2356-2361.)

Key Words: apoptosis ■ cerebral ischemia, focal ■ DNA damage ■ reperfusion injury ■ superoxide dismutase ■ mice

Reperfusion injury is thought to play a critical role in the pathophysiology of cerebral ischemia. Although reperfusion of ischemic tissue with thrombolytic agents after a short period of ischemia reduces infarction volume, reperfusion at a later period exacerbates ischemic brain damage. Reactive oxygen species (ROS) are implicated in reperfusion injury after cerebral ischemia, and antioxidant enzymes are thought to be among the major mechanisms by which the cells counteract the deleterious effect of ROS after cerebral ischemia and reperfusion.1,2 Among the antioxidant enzymes, copper/zinc superoxide dismutase (SOD1), which scavenges superoxide anions in the cytosol, has been shown to be highly protective against ischemia/reperfusion injury after transient focal cerebral ischemia (FCI).3,4 Overexpression of SOD1 in transgenic (Tg) mice resulted in a reduction of infarction volume and edema formation and better neurological outcomes after transient FCI,5 whereas targeted disruption of SOD1 in mutant mice resulted in a marked exacerbation of DNA fragmentation, infarction volume, and edema formation after transient FCI,4 suggesting that SOD1 prevents postischemic apoptosis, thereby reducing cerebral infarction. However, the biochemical cascades underlying this protective role of SOD1 are still uncertain.

Two major pathways have been reported to be involved in apoptosis in vitro. One, Fas (CD95/APO-1), a death receptor located in the plasma membrane, binds to an adapter protein and pro–caspase-8, which results in autoactivation of caspase-8. The caspase-8 then cleaves downstream caspases and results in apoptosis.6,7 The other pathway, the mitochondria, are involved in apoptosis by releasing cytochrome c to the cytoplasm, where it activates caspases; the mitochondrial pathway is independent of the Fas pathway.8 More recently, caspase-8 was shown to be involved not only in the Fas pathway but in the mitochondrial apoptotic pathway by cleaving the cytosolic factor, Bid.9 Although both pathways have been implicated in the pathophysiology of cerebral ischemia, the relation between caspase-8 expression and postischemic apoptosis is still uncertain, except for the data showing that caspase-8 is expressed by different populations of cortical neurons after permanent middle cerebral artery occlusion (MCAO).10
We sought to elucidate the role of reperfusion in postischemic apoptosis by comparing the amount of cytochrome c release, caspase-8 expression, and DNA fragmentation between the ischemic samples after transient FCI and those after permanent FCI. We further investigated the role of ROS production in apoptosis during reperfusion by comparing caspase-8 expression between wild-type (Wt) mice and Tg mice that overexpress SOD1 after transient FCI.

**Materials and Methods**

**SOD1 Tg Mice**

Heterozygous SOD1 Tg mice of the SOD1 TGHS/SF-218–3 strain with a CD-1 background, carrying human SOD1 genes with a 3-fold increase in copper/zinc-SOD, were derived from the founder stock previously described. The SOD1 Tg mice were identified by quantitative demonstration of SOD1 with non-denaturing gel electrophoresis followed by nitroblue tetrazolium staining. There were no phenotypic differences between the SOD1 Tg mice and their Wt littermates.

**FCI**

Adult male CD-1 mice (35 to 40 g) were subjected to both transient and permanent focal ischemia by intraluminal MCA blockade with a nylon suture. The mice were anesthetized with 2.0% isoflurane in 30% oxygen and 70% nitrous oxide given by a face mask. The rectal temperature was controlled at 37°C with a homeothermic blanket. Cannulation of a femoral artery allowed the monitoring of blood pressure and arterial blood gas, samples for analysis being taken immediately after cannulation and 10 minutes after occlusion. Blood gas was analyzed with a pH/blood gas analyzer (Chiron Diagnostics Ltd). For the transient model, blood flow was restored by the withdrawal of the nylon suture after 60 minutes of MCAO. For the permanent model, the nylon suture was fixed at the final position with a silk suture.

**Histological Assessment**

The experimental animals were killed 0, 1, 2, 4, 8, and 24 hours after 1 hour of transient FCI or 1, 2, 5, and 25 hours after permanent FCI. The brains were removed, rapidly frozen in -20°C 2-methylbutane, and stored at -80°C. They were sectioned with a cryostat, 20 μm thick from the anterior to the posterior side, and stained with cresyl violet.

**DNA Gel Electrophoresis**

DNA extraction from brain tissue was performed as previously described. The animals were killed 5 and 25 hours after both transient and permanent MCAO. The brain tissue was processed for gel electrophoresis and the DNA bands were analyzed as described.

**Western Blot Analysis**

Protein extraction of both the mitochondrial and cytosolic fractions was performed as described. Exactly 3.6 μg of protein from the cytosolic fraction and 2.2 μg from the mitochondrial fraction were loaded per lane on the gel and blotted to polyvinylidene difluoride membrane. Heat-shocked HeLa cell extract (#LYC-HL101F; StressGen, Victoria, Canada) was used as a positive control for caspase-8.

**Results**

**DNA Laddering Was More Prominent After Transient FCI Than After Permanent FCI**

We extracted genomic DNA from the ischemic brain and the homologous sample from the contralateral side and analyzed them by gel electrophoresis (Figure 1). Intranucleosomal DNA fragmentation was absent from the control tissue in both the transient and permanent FCI samples. A significant amount of DNA laddering appeared at 25 hours, which was not detected at 5 hours, after both transient and permanent FCI. At 25 hours, the characteristic laddering was prominent after transient FCI (lane 4), whereas a strong smear background and less laddering were observed after permanent FCI (lane 8).

**Release of Mitochondrial Cytochrome c by Transient and Permanent FCI**

To examine the role of the mitochondrial pathway during reperfusion-induced apoptotic cell death, we analyzed cytosolic cytochrome c in both transient and permanent FCI. Cytochrome c immunoreactivity was evident as a single band with a molecular mass of 15 kDa of the cytosolic fraction after both transient and permanent FCI, whereas it was barely detected in either the normal control brain or the contralateral brain (Figure 2). After transient FCI, cytosolic cytochrome c

[Figure 1] A, Genomic DNA gel electrophoresis. No DNA ladderizing is observed in contralateral brain (4h C and 24h C). In ischemic brain, intranucleosomal DNA fragmentation appeared from 24 hours after both transient and permanent FCI (24h I). DNA ladderization was prominent in ischemic brain 24 hours after transient FCI (24h I, T-MCAO), whereas a significant smear as well as DNA ladderizing were seen 25 hours after permanent FCI (25h I, P-MCAO). DNA was end-labeled with biotinylated dNTP, electrophoresed on 1.5% agarose gel, transferred to nylon membrane, and visualized by chemiluminescent method. One microgram of DNA was loaded per lane. T-MCAO indicates transient MCAO; P-MCAO, permanent MCAO; C, contralateral; and I, ischemic brain. B, Quantitative analysis of DNA gel electrophoresis observed in A.

**Statistical Analysis**

Data are presented as mean±SD or mean±SEM. Statistical comparisons were made by ANOVA, with StatView software, version 4.0 (Abacus Concepts). A value of P<0.05 was considered statistically significant.
was significantly increased as early as 3 hours after MCAO (1 hour of occlusion plus 2 hours after reperfusion), whereas no cytosolic cytochrome c was detected 2 hours after MCAO (1 hour of occlusion plus 1 hour after reperfusion) (Figures 2A and 3). Permanent FCI resulted in a significant increase of cytosolic cytochrome c as early as 2 hours after MCAO (Figures 2B and 3). The cytosolic accumulation of cytochrome c was sustained until 25 hours after both transient and permanent FCI (Figures 2 and 3). β-Actin showed no alteration after either transient or permanent FCI (Figure 2). A significant amount of mitochondrial cytochrome c was detected in the control mitochondria, and was reduced hours after both transient and permanent FCI.12,18 In contrast, mitochondrial cytochrome oxidase, as a control mitochondrial protein, showed much less alteration in the same samples.12,18

Caspase-8 Is Induced After Transient But Not After Permanent FCI

We analyzed caspase-8 in both transient and permanent FCI. Caspase-8 immunoreactivity was evident as a band with a molecular mass of ≈55 kDa in either the normal control brain or the contralateral brain (Figure 4, A and B), indicating the constitutive expression of its proform in the mouse brain. HeLa cell extracts were used as a positive control and showed strong caspase-8 immunoreactivity with the antibody (Figure 4B, lane 7). Caspase-8 protein expression was apparently increased as early as 5 hours after transient FCI (1 hour of occlusion plus 4 hours of reperfusion) (Figure 4A). The induction of caspase-8 remained until 25 hours after transient FCI (Figure 4A). Permanent FCI resulted in a constant expression of caspase-8, and there was no induction until 25 hours after ischemia (Figure 4B). β-Actin showed no alteration after either transient or permanent FCI (Figure 4, A and B, lower panel). Figure 5 demonstrates the quantitative analysis of caspase-8 protein after transient and permanent FCI. A significant increase in caspase-8 was confirmed 4 hours (4.00-fold compared with control; P=0.005, n=3 and
Caspase-8 Induction After Cerebral Ischemia

Discussion

Several pathways have been reported to be involved in apoptosis in vitro.6–9 We have reported that mitochondrial cytochrome c translocates to the cytosol after FCI.12,14,18 Translocation of caspase-9 from mitochondria to the cytosol is reported after transient global cerebral ischemia.19 In contrast, apoptosis through the Fas pathway is involved in cell death after cerebral ischemia. Fas (CD95/APO-1) is upregulated after cerebral hypoxic-ischemic injury to the developing rat brain.20 Caspase-8 and caspase-3 are expressed by different populations of cortical neurons undergoing delayed cell death after permanent FCI.9 Caspase-3, an effector caspase that can activate DNase during apoptosis,21 is activated after transient FCI.22 These reports suggest the possible role of both mitochondria-mediated and Fas-mediated pathways in postischemic apoptosis. However, the role of reperfusion in these pathways has not been examined, although reperfusion was shown to alter distribution of neuronal apoptosis after FCI.23 Our study revealed the different patterns of DNA fragmentation between the ischemic samples after transient and permanent FCI (Figure 1). We have reported that early release of mitochondrial cytochrome c contributed to apoptosis not only after transient FCI18 but also after permanent FCI.12 In the present study, cytochrome c release was shown in both transient and permanent FCI, whereas DNA fragmentation was more prominent after transient FCI (Figure 1). Therefore, we conducted the comparison of caspase-8 expression, which could promote an alternative apoptotic pathway, between transient and permanent FCI.

Caspase-8 induction was significantly enhanced in transient FCI in contrast to permanent FCI, suggesting that caspase-8 induction during reperfusion contributes to apoptosis after transient FCI, in addition to the cytochrome c pathway. Alternatively, we do not rule out the possibility that ATP depletion after permanent FCI prevents downstream caspase activation and apoptosis, because ATP is required for cytochrome c to trigger the cytosolic downstream event.8

Induction of caspase-8 after transient FCI may be closely related to apoptosis through proteolytic activity of its activated form. Overexpression of pro–caspase-8 induces apoptosis in cells,24,25 whereas downregulation of pro–caspase-8 causes activation-induced resistance to apoptosis in monocytes.26 In fact, caspase-8 is upregulated in cells and then activated, which increases sensitivity to apoptotic signals during apoptosis induced by interferon γ or human immunodeficiency virus type 1 infection.27,28 Pro–caspase-8 mRNA is upregulated after transient spinal cord ischemia, where caspase-8-mediated apoptosis is assumed to participate.29 Pro–caspase-8 is known to be activated in neurons after permanent FCI and spinal cord ischemia,10,29 suggesting the role of the pathway through caspase-8 in apoptosis after FCI.

Figure 6. A, Western blot analysis of caspase-8 4 hours after reperfusion in transient FCI in Wt and SOD1 Tg mice. Caspase-8 was apparently induced in Wt mice, whereas no induction was observed in SOD1 Tg mice. B, Quantitative analysis of caspase-8 expression in Wt and Tg mice.

Physiological Data and Cerebral Infarction

There were no significant differences in mean arterial blood pressure and arterial blood gas analysis between groups. The preischemic physiological values are (transient/permanent FCI): mean arterial blood pressure, 71.50±3.42/74.0±6.32 mm Hg; Pao2, 157.25±20.13/174±5.16 mm Hg; Paco2, 33.05±4.67/32.35±5.76 mm Hg; pH, 7.330±0.063/7.317±0.070 (values are mean±SD, n=4). There was no deviation from these values over the period of assessment. An ischemic lesion of the core of the caudate putamen was visible as a pale, slightly stained area in the ischemic hemisphere as early as 1 hour after FCI and extended to the entire MCA territory at 5 hours by cresyl violet staining (data not shown). The time-dependent increase of the infarction with the intraluminal suture blockade is consistent with previous reports that used the same focal stroke model in mice.4,5,12

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and the occurrence of proteolytic processing of pro-caspase-8 after FCI. Therefore, the induction of pro-caspase-8 after transient FCI observed in the present study may result in increased caspase-8 activity. Further studies with antibodies to detect the activated form of caspase-8 or an activity assay are warranted to address this issue. To investigate the role of caspase-8 on apoptosis after FCI, double staining with caspase-8 and TUNEL would be useful to demonstrate the relation between caspase-8 expression and DNA fragmentation. DNA fragmentation occurs in the cell that expresses caspase-8 after permanent FCI and spinal cord ischemia.10,29 These results support the idea that caspase-8, which is induced by reperfusion, may promote apoptosis after FCI. Furthermore, it would be of great interest to know if SOD1 Tg mice have less cytochrome c release in the same neurons where caspase-8 is not expressed. We have previously shown the anatomic correlation between cytosolic expression of cytochrome c and DNA fragmentation in a single cell level by using double staining with cytochrome c and TUNEL.18 The relation between caspase-8 and cytosolic cytochrome c after FCI remains to be elucidated.

Regarding tissue damage after transient FCI, infarction is significantly reduced in SOD1 Tg mice compared with Wt mice 6 and 24 hours after transient FCI.3,5 SOD1 Tg mice showed a much better neurological outcome after transient FCI compared with Wt mice, as reported previously.5 Taken together with our data, overexpression of SOD1 appears to reduce the early induction of caspase-8 and subsequent apoptosis and infarction at 24 hours, when infarction is completed in our model.4 However, in light of the fact that very delayed expansion of infarction occurs after mild FCI,30 we do not rule out the possibility that delayed expansion of the cerebral infarction could occur in SOD1 Tg mice. Further evaluation of caspase-8 expression, DNA laddering, and infarction volume at later time points is warranted to address this important issue. Nevertheless, SOD1 expression is not modified after transient FCI,18 and we believe that a cumulative effect of SOD1 may also prevent such delayed expansion of infarction.

We have shown that SOD1 reduces mitochondrial cytochrome c release, DNA fragmentation, and infarction volume after transient FCI13,18,31 but not after permanent FCI.32 These results indicate that superoxide radicals are significantly involved in reperfusion injury after FCI. In fact, we previously detected the production of superoxide anions after both focal and global cerebral ischemia by using a hydroethidium injection method.12,33,34 Overexpression of SOD1 resulted in a marked decrease in postischemic superoxide production.34 In addition, we also observed that the level of hydroethidium oxidation was significantly reduced in SOD1 Tg mice during reperfusion after photothrombotic stroke, compared with Wt mice.35 Therefore, we believe that SOD1 Tg mice have less superoxide production during reperfusion. Protein expression of caspase-8 was significantly induced after FCI (Figures 4 and 5) and was reduced by SOD1 overexpression 4 hours after transient FCI (Figure 6). Therefore, we suggest that superoxide production during reperfusion contributes to apoptosis through caspase-8 induction. Alternatively, it would be of interest to perform double staining with hydroethidium and cytochrome c, caspase-8, and TUNEL after transient FCI. The detailed mechanism by which superoxide radical induces caspase-8 is unclear. Because ROS were reported to be closely associated with the acute activation of transcription factors such as AP-1 and NF-κB,30 it is conceivable that ROS activate a certain transcription factor, thereby inducing caspase-8 after transient FCI. These issues remain to be elucidated in a future study.

It would be interesting to follow DNA damage in single cells by using markers of DNA damage and repair. Using the same model, we have demonstrated that SOD1-null mice showed a marked increase in DNA fragmentation as shown by TUNEL staining.4 Moreover, SOD1 Tg mice had less reduction in the expression of the DNA repair enzyme, apurinic/apyrimidinic endonuclease (APE), which plays a central role in repairing DNA damage after oxidative stress after transient FCI. Interestingly, DNA fragmentation occurs in neurons that lack APE expression, suggesting that an early decrease in APE and the failure of the DNA repair mechanism contribute to DNA damaged cell death after transient FCI.32

In summary, we found that DNA fragmentation was more prominent in brains after transient FCI than after permanent FCI. Caspase-8, which plays a major role in both Fas-dependent and cytochrome c-dependent apoptotic pathways, was significantly induced after transient FCI but not after permanent FCI. Caspase-8 induction was reduced in Tg mice that overexpress SOD1 compared with Wt mice after transient FCI. The results suggest that increased ROS production during reperfusion may contribute to the induction of caspase-8 and could thereby exacerbate apoptosis after FCI. Caspase-8 could be a therapeutic target against reperfusion injury in a clinical situation, such as acute thrombolytic therapy for embolic stroke or temporary occlusion of the main arteries during cerebrovascular surgery.

Acknowledgments

This study was supported by National Institutes of Health grants NS14543, NS25372, NS36147, NS38653, and NO1-NS82386. Dr Chan is a recipient of the Jacob Javits Neuroscience Investigator Award. We are grateful to Dr Charles J. Epstein, Department of Pediatrics, University of California, San Francisco, School of Medicine, for continuous collaboration by providing breeding pairs of SOD1 transgenic mice. We thank Dr Jean-Christophe Copin for the organization of genotyping transgenic animals. We also thank Jane O. Kim for assistance with the Western blot analysis, Liza Reola and Bernard Calagui for technical assistance, and Cheryl Christensen for editorial assistance.

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Stroke. 2001;32:2356-2361
doi: 10.1161/hs1001.097241

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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