Copper-Zinc Superoxide Dismutase Affects Akt Activation After Transient Focal Cerebral Ischemia in Mice

Nobuo Noshita, MD; Taku Sugawara, MD, PhD; Anders Lewén, MD, PhD; Takeshi Hayashi, MD, PhD; Pak H. Chan, PhD

Background and Purpose—The serine-threonine kinase Akt is activated by phosphorylation at serine-473. After phosphorylation, activated Akt inactivates BAD or caspase-9 or other apoptogenic components, thereby inhibiting cell death. In this study we examined the relationship between Akt phosphorylation and oxidative stress after transient focal cerebral ischemia (FCI) using copper-zinc superoxide dismutase (SOD1) transgenic (Tg) mice.

Methods—The mice were subjected to 60 minutes of middle cerebral artery occlusion by intraluminal suture blockade followed by 1, 4, and 24 hours of reperfusion. Phospho-Akt expression was examined by immunohistochemistry and Western blot analysis. Production of superoxide anion was assessed by the hydroethidine method in both wild-type mice and SOD1 Tg mice. DNA fragmentation was evaluated by terminal deoxynucleotidyl transferase–mediated uridine 5'-triphosphate-biotin nick end labeling (TUNEL).

Results—Immunohistochemistry demonstrated that phospho-Akt was constitutively expressed and was decreased in the ischemic core as early as 1 hour after reperfusion, whereas it was temporally increased in the cortex at 4 hours. Phospho-Akt expression was enhanced in the SOD1 Tg mice. Western blot analysis showed that phospho-Akt was maximized 4 hours after reperfusion in the wild-type mice, whereas phospho-Akt was increased as early as 1 hour after ischemia in the SOD1 Tg mice. There was a significant decrease in TUNEL-positive cells in the SOD1 Tg mice compared with the wild-type mice.

Conclusions—The present study suggests that SOD1 may contribute to the early activation of the Akt cell survival signaling pathway and may attenuate subsequent DNA damage after transient FCI. (Stroke. 2003;34:1513-1518.)

Key Words: apoptosis ■ cerebral ischemia, focal ■ reactive oxygen species ■ superoxide dismutase ■ mice

The serine-threonine kinase Akt, which is also referred to as protein kinase B and RAC, is the cellular homologue of the viral oncogene v-Akt. This protein kinase plays a critical role in controlling the balance between survival and apoptosis in cells. Akt is activated by insulin and various growth and survival factors and functions in a wortmannin-sensitive pathway involving phosphatidylinositol 3-kinase (PI3-kinase). Akt contains an amino-terminal pleckstrin homology domain, which binds phosphorylated lipids at the membrane in response to activation of PI3-kinases. Akt is activated by phospholipid binding and activation-loop phosphorylation at threonine-308 by phosphatidylinositol (3,4,5)-trisphosphate-deinase 13 and also within the C-terminus at serine-473 by integrin-linked kinase.4 After phosphorylation, Akt promotes cell survival and prevents apoptosis by inactivating several targets, including BAD,5,6 glycogen synthase kinase-3,7 forkhead transcription factors,8 or caspase-9.9 Changes in phospho-Akt levels were reported after global cerebral ischemia10,11 and focal cerebral ischemia (FCI)12,13 showing a temporal increase in phospho-Akt at serine-473. There are 3 isoforms of Akt (Akt1, Akt2, Akt3). Although the type of organs that express each enzyme is different, all of these isoforms are highly expressed in the brain.14

The antioxidant enzyme is one of the major mechanisms by which cells counteract the deleterious effects of reactive oxygen species (ROS), and recent studies have revealed the protective effect of antioxidant enzymes on apoptosis after cerebral ischemia and reperfusion.18,19 We have shown that superoxide dismutase (SOD) plays a protective role against FCI,14–17 as well as global cerebral ischemia.18,19 These reports show that SOD is involved in regulation of cellular damage after ischemia/reperfusion.

Recent in vitro studies have shown that Akt activation is induced by ROS20–22 or nitric oxide.23 However, a role for antioxidants like copper-zinc SOD (SOD1) on the cell survival signaling pathway involving Akt in FCI has not been elucidated. In this study we address this issue by examining Akt phosphorylation after transient FCI, using both wild-type mice and SOD1 transgenic (Tg) mice.
Materials and Methods

Focal Cerebral Ischemia

All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Stanford’s Administrative Panel on Laboratory Animal Care. 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 SOD1, were derived from the founder stock described previously.24 They were further bred with CD-1 wild-type mice to generate heterozygous mice. Adult male SOD1 Tg mice and non-Tg littermates (weight, 35 to 40 g; age, 3 months; n=44 each) were subjected to transient FCI by intraluminal middle cerebral artery (MCA) blockade with a nylon suture, as previously described.16 There were no differences in phenotypes, including the anatomy of the circle of Willis, between the SOD1 Tg mice and their littermates.26 There was no difference in the regional cerebral blood flow before or after FCI between the SOD1 Tg mice and the wild-type mice.22 Thus, vascular anatomy and cerebral blood flow measurements were not considered in the present experiments. The mice were anesthetized with 1.5% isoflurane in 30% oxygen and 60% nitrous oxide with the use of a face mask. After a midline skin incision, the left external carotid artery was exposed, and its branches were electrocoagulated. An 11-mm 5-0 surgical monofilament nylon suture, blunted at the end, was introduced into the left internal carotid artery through the external carotid artery stump. After 60 minutes of MCA occlusion, blood flow was restored by the withdrawal of the nylon suture.

Immunohistochemistry of Phospho-Akt

Anesthetized animals were perfused with 200 mL of 10 U/mL heparin in 0.9% saline and 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) after 1, 4, and 24 hours of reperfusion, as well as normal controls (n=3). Brains were removed, postfixed for 12 hours in 4% paraformaldehyde, sectioned at 50 μm with a vibrotome, and processed for immunohistochemistry. The sections were incubated with blocking solution and reacted with rabbit polyclonal anti-sOD1 (serine-473) antibody IHC Specific (for immunohistochemistry; Cell Signaling) at a dilution of 1:200. Immunohistochemistry was performed with the avidin-biotin technique, and then the nuclei were counterstained with methyl green solution for 10 minutes.

In Situ Detection of Superoxide Anion Production

Early production of superoxide anion (O2−) in cerebral ischemia was investigated with the use of hydroethidine as previously described.14 Hydroethidine is diffusible into the central nervous system parenchyma after an intravenous injection and is selectively oxidized to ethidium by O2− but not by other ROS such as hydrogen peroxide, hydroxyl radical, or peroxynitrite.14 Hydroethidine solution (200 μL; 1 mg/mL in PBS) was administered intravenously 15 minutes before induction of ischemia as described.14 In the brains of animals intravenously injected with hydroethidine, fluorescence was assessed microscopically at excitation=510 nm and emission >580 nm for ethidium detection. The animals were killed 1 hour after transient FCI by transcardial perfusion. After fixation with 4% paraformaldehyde for 12 hours, the brains were sectioned at 50 μm on a vibratome. Subsequently, the slides were covered with VECTASHIELD mounting medium with 4',6 diamidino-2-phenylindole (DAPI) (Vector Laboratories). Fluorescence of DAPI was also observed at excitation = 360 nm and emission >460 nm.

Western Blot Analysis

Whole cell protein extraction was performed. Samples were obtained from the entire MCA territory on the ischemic sides and from nonischemic controls (n=6 each). Fresh brain tissue was cut into pieces after 1, 4, and 24 hours of reperfusion and homogenized in 7 volumes of cold suspension buffer as previously described.15 The homogenate was centrifuged at 10 000g for 20 minutes at 4°C, and the supernatant was used for the analysis. After the same volume of Tris-glycine sodium dodecyl sulfate sample buffer (Invitrogen) was added to the supernatant, equal amounts of the samples were loaded per lane. The primary antibodies were 1:1000 dilution of rabbit polyclonal antibody against phospho-Akt (serine-473) and Akt (Cell Signaling) or 1:10 000 dilution of anti-β-actin monoclonal antibody (Sigma). Western blots were performed with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Cell Signaling) or anti-mouse immunoglobulin G (Chemicon International) with the use of enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences). The film was scanned with a GS-700 imaging densitometer (Bio-Rad), and the results were quantified with Multi-Analyt software (Bio-Rad).

In Situ Labeling of DNA Fragmentation

To assess DNA damage, we performed terminal deoxynucleotidyl transferase-mediated deoxyuridine 5’-triphosphate-biotin nick end labeling (TUNEL) after transient FCI in both wild-type mice (n=4) and SOD1 Tg mice (n=4), as previously described.28 Staining was visualized with the use of 0.025% diaminobenzidine, 0.075% H2O2, and 1% nickel sulfate in PBS, followed by methyl green staining.

Quantification and Statistical Analysis

To analyze the amount of phospho-Akt and β-actin, we used optical density as the unit. The data are expressed as mean±SD. To quantify the DNA-fragmented cells after ischemia, the number of TUNEL-positive cells in the caudate putamen and the ischemic cortex was counted at 24 hours of reperfusion in a high-powered field (×400) by an investigator who was blinded to the studies and was expressed as number per square millimeter. To assess the expression of phospho-Akt in Western blot analysis, we performed 1-factor ANOVA followed by Fisher’s protected least significant difference test for multiple-group analysis. To assess the number of TUNEL-positive cells, we performed the Student’s t test for 2-group analysis. P<0.05 was considered statistically significant.

Results

Phospho-Akt Expression After Transient FCI in Wild-Type and SOD1 Tg Mice

Phospho-Akt (serine-473) was constitutively expressed in the entire region of the normal mouse brain, including the caudate putamen (Figure 1A) and the cortex (Figure 1B). After 1 hour of reperfusion, the expression of phospho-Akt was reduced in the caudate putamen (Figure 1C). This reduction was sustained until 24 hours of reperfusion (Figure 1E, 4 hours; Figure 1G, 24 hours). In contrast, phospho-Akt expression was increased at 4 hours of reperfusion in the ischemic cortex (Figure 1F). After 24 hours of reperfusion (Figure 1H), immunoreactivity of phospho-Akt was diminished compared with the cortex in the control. To assess the role of ROS on Akt phosphorylation, we examined phospho-Akt expression in the SOD1 Tg mice 1 hour after reperfusion. In the caudate putamen, phospho-Akt was reduced in the wild-type mice (Figure 1C), whereas the reduction was not obvious in the SOD1 Tg mice (Figure 1I). In the ischemic cortex, phospho-Akt expression was enhanced in the SOD1 Tg mice (Figure 1J), while phospho-Akt expression was not increased in the wild-type mice 1 hour after FCI (Figure 1D) compared with the normal control.

Production of O2− After FCI in Wild-Type and SOD1 Tg Mice

O2− production was shown by oxidized hydroethidine signals (red) in the cytosol of the contralateral cortex in both the wild-type (Figure 2A) and SOD1 Tg mice (Figure 2B);
however, no conspicuous difference was observed between them. In the ischemic cortex of the wild-type mice, enhanced vesicular signals were observed (Figure 2C, arrowheads), which were markedly decreased in the SOD1 Tg mice (Figure 2D) compared with the wild-type mice, suggesting inhibition of O$_2^-$ production by SOD1.

Western Blot Analysis of Phospho-Akt and Akt After Transient FCI

As shown in Figure 3, phospho-Akt and Akt were evident as a band of 60 kDa in the whole cell fraction from the mouse brains. In the MCA territory lesion, phospho-Akt was constitutively expressed in the nonischemic control brain. Phospho-Akt was significantly increased in the wild-type mice 4 hours after reperfusion (*$P<0.05$) compared with the nonischemic control. In the SOD1 Tg mice, phospho-Akt was significantly increased 1 and 4 hours after reperfusion compared with the nonischemic control ($\dagger P<0.005$, **$P<0.0005$). Furthermore, phospho-Akt was significantly increased 1 hour after reperfusion in the SOD1 Tg mice compared with the wild-type mice ($\#P<0.005$). Akt did not show a prominent modification after reperfusion. These data suggest that phosphorylation of Akt is temporarily accelerated after transient FCI and that SOD1 contributes to Akt phosphorylation.

In Situ Labeling of DNA Fragmentation After Transient FCI in Wild-Type and SOD1 Tg Mice

To evaluate apoptotic cell death, we performed TUNEL staining 24 hours after transient FCI. TUNEL-positive cells with shrunken, darkly stained nuclei and apoptotic bodies were observed in the entire MCA territory in the wild-type...
mice (Figure 4A, caudate putamen; Figure 4B, ischemic cortex), whereas TUNEL-positive cells were seen mainly in the caudate putamen in the SOD1 Tg mice (Figure 4C). Quantitative analysis (n=4) showed a significant decrease in TUNEL-positive cells in the SOD1 Tg mice compared with wild-type mice (Figure 4E; P<0.05; wild-type, 1.48±0.19×10^3 cells/mm²; SOD1 Tg, 1.06±0.22×10^3 cells/mm²) and the cortex (Figure 4F; P<0.05; wild-type, 0.99±0.70×10^3 cells/mm²; SOD1 Tg, 0.02±0.02×10^3 cells/mm²).

**Discussion**

Akt activation is one of the principal factors that prevent apoptosis in many cellular systems. Several studies have shown Akt phosphorylation against various stimuli both in vitro and in vivo. Our recent study revealed that Akt phosphorylation is temporally increased in the ischemic cortex after transient FCI and that inhibition of Akt phosphorylation by a PI3-kinase inhibitor accelerates DNA fragmentation in the ischemic cortex after ischemia, suggesting that Akt is involved in the cell survival signaling pathway after transient FCI. In the present study we examined the relationship between ROS and Akt activation after transient FCI, using SOD1 Tg mice.

Our results show that the increase in phospho-Akt at serine-473 after transient FCI was seen earlier in the SOD1 Tg mice than in the wild-type mice. Immunohistochemistry showed that phospho-Akt expression was decreased in the...
ischemic core of the wild-type mice as early as 1 hour after reperfusion (Figure 1G) and that the decrease was sustained until 24 hours (Figure 1E). Phospho-Akt expression was enhanced in the ischemic cortex 4 hours after reperfusion (Figure 1D). However, phospho-Akt expression was not decreased in the ischemic core of the SOD1 Tg mice 1 hour after reperfusion (Figure 1I). Moreover, phospho-Akt expression was increased in the ischemic cortex of the SOD1 Tg mice as early as 1 hour after reperfusion (Figure 1J), suggesting that Akt phosphorylation was increased in both the caudate putamen and the ischemic cortex of the SOD1 Tg mice compared with the wild-type mice. The regional difference in Akt activation after FCI has already been reported, and it is conceivable that the difference in Akt phosphorylation might depend largely on the severity of the stress. Excessively damaged lesions like those in the ischemic core can decrease Akt phosphorylation due to cell death, whereas a moderately damaged area such as in the ischemic cortex can transiently increase Akt phosphorylation. Excessive damage can increase dephosphorylation of Akt without a transient increase in Akt phosphorylation. From the present results, SOD1 may attenuate cellular damage after FCI and inhibit dephosphorylation of Akt. This is supported by the results of the Western blot analysis, which showed that phospho-Akt was decreased shortly after reperfusion followed by a transient increase at 4 hours in the MCA lesion in the wild-type mice (Figure 3), whereas phospho-Akt was increased as early as 1 hour after reperfusion in the SOD1 Tg mice (Figure 3). Furthermore, dephosphorylation of Akt immediately after an insult has also been observed after global ischemia. When these findings are considered, SOD1 may have an effect in decreasing the initial dephosphorylation of Akt shortly after the insult. Since SOD1 can reduce O$_2^-$ production compared with the wild-type mice (Figure 2), a decrease in O$_2^-$ production might attenuate dephosphorylation of Akt and decrease subsequent DNA fragmentation after FCI (Figure 4). The results mean that mild O$_2^-$ production can increase Akt phosphorylation, even though excessive O$_2^-$ production accelerates dephosphorylation of Akt. In vitro studies showed that ROS could cause Akt activation. Akt phosphorylation was temporally increased in cultured myocytes after exposure to H$_2$O$_2$. In vascular smooth muscle cells, Akt phosphorylation was increased by H$_2$O$_2$ stimulation. Moreover, it has been shown that ROS stimulate Akt phosphorylation in C$_6$C$_{12}$ skeletal myotubes. These reports indicate that ROS are one of the triggers for Akt activation, but the mechanism is still unclear. We do not have data to suggest a direct effect of O$_2^-$ on Akt phosphorylation. We speculate that a decrease in the O$_2^-$ level and thus a decrease in oxidative stress (redox state) in the cell may act as a molecular switch that affects redox-sensitive proteins/enzymes, such as nuclear factor-$kappa$B, that may have an effect on growth factor P73K/Akt signaling and subsequent cell survival/death after ischemia.

Previous studies have revealed the protective effect of SOD1 in ischemic damage. We have shown that overexpression of SOD1 reduced neuronal injury and DNA fragmentation in the hippocampal CA1 subregion after global cerebral ischemia. Moreover, mitochondrial cytochrome $c$ release and subsequent caspase activation were also attenuated by SOD1, suggesting a protective role in neuronal damage after global cerebral ischemia. In a transient FCI model, we have shown that overexpression of SOD1 decreased infarct volume, prevented mitochondrial cytochrome $c$ release, reduced the loss of a DNA repair protein, apurinic/apyrimidinic endonuclease, downregulated nuclear factor-$kappa$B and c-Myc expression, and attenuated acute activation of activator protein-1. These studies suggest that SOD1 inhibits the deleterious effect of ROS in ischemia/reperfusion by multiple and interrelated pathways.

In conclusion, we have shown that overexpression of SOD1 in Tg mice reduces O$_2^-$ production and enhances Akt activation after transient FCI by preventing excessive damage from O$_2^-$, which might contribute to decreasing DNA fragmentation.

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