Modulation of Proline-Rich Akt Substrate Survival Signaling Pathways by Oxidative Stress in Mouse Brains After Transient Focal Cerebral Ischemia

Atsushi Saito, MD; Takeshi Hayashi, MD, PhD; Shuzo Okuno, MD; Tatsuro Nishi, MD, PhD; Pak H. Chan, PhD

Background and Purpose—A proline-rich Akt substrate (PRAS) contributes to the regulation of apoptosis after a variety of cell death stimuli, as well as in an in vivo transient focal cerebral ischemia (tFCI) model. We reported previously that overexpression of copper/zinc-superoxide dismutase (SOD1) reduces apoptotic cell death after tFCI. Our present study was designed to clarify the relationship between the PRAS signaling pathway and oxidative stress in the regulation of apoptosis after tFCI.

Methods—We used a tFCI model with SOD1 transgenic mice and wild-type littermates to examine the expression of phosphorylated PRAS (pPRAS) by Western blotting and immunohistochemistry and the interaction of pPRAS with phosphorylated Akt (pPRAS/pAkt) or the 14-3-3 protein (pPRAS/14-3-3) by coimmunoprecipitation. Direct oxidation of the carbonyl groups, an indication of oxidative injury to total and individual proteins caused by tFCI, was examined using a 2,4-dinitrophenylhydrazone reaction assay.

Results—Expression of pPRAS, pPRAS/pAkt, and pPRAS/14-3-3 decreased 2 hours after tFCI. Oxidized hydroethidine did not colocalize with expression of pPRAS. Individual oxidized carbonyls in pPRAS remarkably increased 2 hours after tFCI but were significantly reduced by SOD1 2 hours after tFCI. Expression of pPRAS, pPRAS/pAkt, and pPRAS/14-3-3 was promoted by SOD1 during the same time course.

Conclusions—These results suggest that overexpression of SOD1 may affect the PRAS pathway after tFCI by reducing the direct oxidative reaction to pPRAS after reperfusion injury. (Stroke. 2006;37:513-517.)

Key Words: apoptosis ■ cerebral ischemia

The apoptotic pathway is regulated by a variety of factors and is based on the balance between cell death and survival factors.1 Serine/threonine kinases, such as Akt (protein kinase B), are key regulators of neuronal cell death and survival after cerebral ischemia.2 Akt functions as a major downstream target of the phosphatidylinositol 3-kinase (PI3-K) pathway, and after the phosphorylation of Akt, it phosphorylates many substrates on the serine or threonine residue, including glycogen synthase kinase-3, Caenorhabditis elegans DAF-16 transcription factor, Bad, phosphodiesterase 3B, and Forkhead transcription factor (FKHR).3–7 In apoptotic neuronal cell death after in vivo cerebral ischemia, Akt is regulated downstream of the PI3-K pathway and regulates substrates such as phosphorylated Bad at serine-136, serine-155 and FKHR.8,9

A proline-rich Akt substrate (PRAS) was purified, sequenced, and identified as a proline-rich molecule by Kovacina et al,10 who also demonstrated that PRAS can be phosphorylated by purified Akt, that the activation of an inducible Akt/mer-Akt is alone sufficient to induce PRAS phosphorylation, that PRAS phosphorylation decreases in cells that lack Akt1 and Akt2, and that phosphorylation of this protein leads to its binding to the 14-3-3 protein. We examined the specificity of an antibody for phosphorylated PRAS (pPRAS). We have also found that pPRAS plays an important role in cell survival during apoptotic neuronal cell death after in vivo cerebral ischemia.11

Reactive oxygen species have been implicated in the mechanism of reperfusion injury after cerebral ischemia.12,13 The electron flow in isolated brain mitochondria produces superoxide anions, which are scavenged by superoxide dismutase (SOD).14 We have shown that copper/zinc-SOD (SOD1), a cytosolic isoenzyme, is highly protective against ischemia and reperfusion injury after transient focal cerebral ischemia (tFCI).9,14,15 Our in vivo studies have demonstrated that SOD1 promotes the cell survival pathway by activating regulators in the PI3-K signaling pathway in ischemia models.9,15,16 However, whether SOD1 affects PRAS remains...
unknown. The present study was designed to clarify the role of SOD1 in the PRAS pathway after tFCI.

Materials and Methods

SOD1 Transgenic Mice
Heterozygous SOD1 transgenic (Tg) mice of the SOD1 TGHS/SF-218-3 strain with a CD-1 background and carrying human SOD1 genes with a 3-fold increase in SOD1, were derived from the founder stock described previously.17 There were no differences in the phenotypes or in the regional cerebral blood flow before or after focal cerebral ischemia between the SOD1 Tg mice and their wild-type littermates.

Focal Cerebral Ischemia
Adult male mice (3 months of age; 35 to 40 g) were subjected to tFCI by intraluminal middle cerebral artery blockade with a 6-0 nylon suture as described previously.13,18 The mice were anesthetized with 1.5% isoflurane in 30% oxygen and 70% nitrous oxide using a face mask. After 60 minutes of middle cerebral artery occlusion, blood flow was restored by withdrawal of the nylon suture.

Western Blot Analysis
Protein extraction was performed to obtain the mitochondrial and cytosolic fractions as described previously.19 Equal amounts of the samples were loaded per lane. The primary antibodies were 1:600 dilution of an antibody against pPRAS (BioSource International), 1:600 dilution of an antibody against phosphorylated Akt (pAkt; Cell Signaling Technology) and Akt (Cell Signaling Technology), or 1:10000 dilution of an anti–β-actin monoclonal antibody (Sigma-Aldrich). Western blots were performed with horseradish peroxidase–conjugated immunoglobulin G (Cell Signaling Technology) and Akt (Cell Signaling Technology), or a 1:600 dilution of an antibody against pPRAS (BioSource International), and 1:600 dilution of an antibody against phosphorylated Akt (pAkt; Cell Signaling Technology) and Akt (Cell Signaling Technology), or 1:10000 dilution of an anti–β-actin monoclonal antibody (Sigma-Aldrich). Western blots were performed with horseradish peroxidase–conjugated immunoglobulin G (Cell Signaling Technology) with the use of enhanced chemiluminescence detection reagents (Amersham International).

Coimmunoprecipitation
Protein extraction and immunoprecipitation were performed as described previously.9,20,21 A total of 200 μg of the protein sample was incubated with protein G-Sepharose (Amer sham Pharmacia Biotech) and with an anti-pPRAS antibody (BioSource International) or an anti-2,4-dinitrophenylhydrazone (DNP) antibody (Chemicon International) for 3 hours at 4°C. A positive control was the whole protein sample, and a negative control lacked an antibody. The pellets were used as the samples and were immunoblotted with the use of an anti-pAkt antibody (Cell Signaling Technology) or an anti–14-3-3 antibody (Cell Signaling Technology) as described in the Western blot analysis.

Immunofluorescent Double Labeling With pPRAS and Oxidized Hydroethidine/TUNEL
Animals were killed by tran cardial perfusion, and the brains were sectioned at 50 μm on a vibratome (n=4 each). The sections fixed by 4% paraformaldehyde were immunostained with specific antibodies (Amersham International). Immunofluorescent double staining demonstrated that strong oxidized HEt expression did not colocalize with expression of pPRAS in the ischemic penumbral cortical lesion area 2 hours after tFCI (Figure 2A and 2B). Oxidized HEt decreased and pPRAS increased in the SOD1 Tg mice compared with the wild-type mice (Figure 2A and 2B). Most TUNEL-immunoreactive cells did not colocalize with expression of pPRAS in the ischemic cortical lesion 24 hours after tFCI (Figure 3A and 3B). TUNEL immunopositivity decreased and pPRAS increased in the SOD1 Tg mice compared with the wild-type mice (Figure 3A and 3B). These results suggest that pPRAS may decrease in cells in which superoxide is strongly produced and in which the apoptotic reaction remarkably progresses in the ischemic penumbral lesion after tFCI.

Detection of Oxidized Carbonyls in Proteins
A commercial detection assay was used with DNP to examine direct oxidative injury to proteins (Chemicon International). The methodological protocols of the manufacturer were followed.22 DNP specifically reacted with the oxidized carbonyl residue on the protein samples.23–25 The DNP binding sites of the oxidized proteins were specifically detected using an anti-DNP antibody by Western blot or coimmunoprecipitation.22,23,26

Quantitative and Statistical Analysis
The data are expressed as mean±SD. Comparisons among multiple groups were performed using 1-way ANOVA with Fisher post hoc tests (SigmaStat software; Jandel Corporation). Comparisons between 2 groups were achieved using Student’s t test. Significance was accepted with P<0.05.

Results
In our previous study, we reported on pPRAS and the binding of pPRAS to pAkt (pPRAS/pAkt) and to the 14-3-3 protein (pPRAS/14-3-3) after tFCI by Western blot analysis.11 All of these expressions transiently decreased during the early period of reperfusion injury after tFCI and significantly decreased 2 hours after tFCI.11 Immunohistochemistry revealed that pPRAS expression was observed in neuronal cells in the cortical area after tFCI (Figure 1). It transiently decreased 2 hours after tFCI compared with the control sample and recovered 24 hours after tFCI (Figure 1). This time course of pPRAS was in accord with our previous results from Western blot analysis.11

Immunofluorescent double staining demonstrated that strong oxidized HEt expression did not colocalize with expression of pPRAS in the ischemic penumbral cortical lesion area 2 hours after tFCI (Figure 2A and 2B). Oxidized HEt decreased and pPRAS increased in the SOD1 Tg mice compared with the wild-type mice (Figure 2A and 2B). Most TUNEL-immunoreactive cells did not colocalize with expression of pPRAS in the ischemic cortical lesion 24 hours after tFCI (Figure 3A and 3B). TUNEL immunopositivity decreased and pPRAS increased in the SOD1 Tg mice compared with the wild-type mice (Figure 3A and 3B). These results suggest that pPRAS may decrease in cells in which superoxide is strongly produced and in which the apoptotic reaction remarkably progresses in the ischemic penumbral lesion after tFCI.

We examined oxidative injury to the individual proteins, indicated by oxidation of the carbonyl groups, with the use of samples precipitated by a DNP antibody after DNP binding. We then confirmed equal amounts of the precipitated proteins among all samples. pPRAS in the proteins bound to DNP (pPRAS/DNP) remarkably increased 2 hours after tFCI.
Expression of pPRAS/DNP significantly decreased in the SOD1 Tg mice compared with the wild-type mice 2 hours after tFCI (Figure 4B; \( *P < 0.05 \)). There was no significant difference between the control samples (Figure 4B). These results suggest that the peak time point of direct oxidative injury to pPRAS was in accord with that of the transient decrease in pPRAS after tFCI.

Western blot analysis revealed that pPRAS, pPRAS/pAkt, and pPRAS/14-3-3 increased in the SOD1 Tg mice compared with the wild-type mice 2 hours after reperfusion (Figure 5A and 5B; \( *P < 0.05 \)). There was no significant difference between the control samples (Figure 5A and 5B). These results suggest that overexpression of SOD1 promoted pPRAS and its interaction with pAkt and 14-3-3 after tFCI.

**Discussion**

PRAS is a novel Akt substrate that was purified, sequenced, and identified by Kovacina et al\(^{10} \) as a proline-rich molecule. These investigators demonstrated that PRAS is phosphorylated by purified Akt, that the activation of an inducible Akt/mer-Akt is alone sufficient to induce PRAS phosphorylation, that PRAS phosphorylation decreases in cells that lack Akt1 and Akt2, and that phosphorylation of this protein leads to its binding to 14-3-3.\(^{10} \) In a previous study, our group demonstrated that pPRAS plays an important role in regulation of cell survival downstream of the PI3-K/Akt pathway after reperfusion injury after tFCI.\(^{11} \)

Protein oxidation introduces the carbonyl groups at the lysine, arginine, proline, and threonine residues. Reaction with 2,4-dinitrophenylhydrazine causes their derivatization to DNP.\(^{24} \) Oxidative modification of proteins can modulate their biochemical characteristics such as enzymatic activity, DNA binding activity of transcription factors, and the susceptibility...
Our previous studies demonstrated that SOD1 overexpression has protective effects against ischemic damage.\(^9,12,13,15\) Superoxide production was detected during the early period of reperfusion in our model.\(^9\) We reported that SOD1 overexpression attenuated apoptotic neuronal cell death by inhibiting proapoptotic regulators, such as released cytochrome c and second mitochondria-derived activator of caspase/direct inhibitor-of-apoptosis protein binding protein with low pH value for the isoelectric point (pI), after reperfusion injury after cerebral ischemia.\(^13,30\) Moreover, our studies revealed that superoxide production may also affect the cell survival pathway and that expression of pAkt and another Akt substrate, Bad, was affected by SOD1 overexpression after tFCI.\(^9,17\) In particular, pAkt expression in the ischemic penumbral lesion was significantly promoted by SOD1 overexpression after tFCI.\(^17\) This may cause upregulation of the Akt pathway downstream. However, in the present study, we focused on oxidative injury to the individual proteins by examining oxidized carbonyl formation in them. Superoxide may diffusely spread in the intracellular space and nonspecifically damage lipid cell membranes and protein enzymes, causing their structural changes in neuronal cells after ischemic insult in vitro.\(^31\) The precise mechanisms of how superoxide may spread and how it may be directly involved with apoptosis regulators are still unknown; however, our results suggest that not only an upstream substrate, but also individual downstream substrates, might be directly injured by oxidative stress and that individual proteins injured by oxidative stress, such as during reperfusion after tFCI, might be rescued by SOD. The relationship between oxidative stress and the effects on oxidized pPRAS was based on the level of pPRAS/DNP in our in vivo model; however, in vitro studies, such as a cell-free assay, are required to strengthen cause and effect between PRAS oxidation and phosphorylation of PRAS. In addition, oxidation and subsequent degradation may also affect the level of total PRAS, which, in turn, may affect the level of pPRAS in the ischemic brain. Because we did not measure the total levels of PRAS after ischemia, our data regarding the relationship between pPRAS and direct oxidation need to be interpreted with caution. Nevertheless, the peak time point for the transient decrease in pPRAS and its complexes was in accord with the transient increase in oxidized pPRAS, and both were significantly affected in the SOD1 Tg mice compared with the wild-type mice. These results show that superoxide may directly or indirectly cause protein oxidation and that it may also affect pPRAS binding to other proteins. Overexpression of pPRAS plays an important role in neuroprotection after tFCI.\(^11\) In contrast, because SOD1 overexpression may directly affect Akt, other kinases, and phosphatases, the relationship between PRAS oxidation and phosphorylation and SOD1 overexpression in neuroprotection in the ischemic brain needs to be further elucidated. Our finding that inhibition of oxidative injury promotes pPRAS may contribute to clarifying the complicated mechanisms of the neuroprotective role of SOD. It may also contribute to developing a new strategy for the treatment of cerebral ischemia.

Figure 5. A, Western blotting demonstrated that pPRAS expression was significantly increased in the SOD1 Tg mice compared with the wild-type mice at the same time point (top row; \(P<0.05\)). \(\beta\)-Actin was used as an internal control for the cytosolic fraction, and no difference was seen between the samples (second row). Coimmunoprecipitation revealed that pPRAS/pAkt expression was significantly increased in the SOD1 Tg mice compared with the wild-type mice 2 hours after tFCI (third row; \(P<0.05\)). Coimmunoprecipitation demonstrated that pPRAS/14-3-3 expression was significantly increased in the SOD1 Tg mice compared with the wild-type mice 2 hours after tFCI (bottom row; \(P<0.05\)). B, OD of these expressions was quantified (\(P<0.05\)). Graphs show mean values (±SD).

to proteolytic degeneration.\(^27-29\) DNP is detected by a specific antibody.\(^24,26\) Using a DNP assay, we demonstrated the following in our previous study. Direct oxidative injury to the cytosolic proteins increased from 1 hour after tFCI but was reduced by SOD1 overexpression until 24 hours after tFCI.\(^22\) Formation of oxidized carbonyls in some regulators involved in apoptotic neuronal cell death was detected during the early period of reperfusion injury, and these oxidized carbonyl proteins were all significantly reduced by SOD1 overexpression after tFCI.\(^22\) In the present study, we showed the following: (1) Formation of oxidized carbonyls in pPRAS was detected during the early period of reperfusion injury; (2) The peak time point of oxidized carbonyls in pPRAS was in accord with a significant decrease in pPRAS, pPRAS/pAkt, and pPRAS/14-3-3 after tFCI; (3) Formation of oxidized carbonyls in pPRAS significantly decreased with SOD1 overexpression at the same time point; (4) Expression of pPRAS, pPRAS/pAkt, and pPRAS/14-3-3 was significantly promoted by SOD1 overexpression after tFCI.
In conclusion, SOD1 contributes to the inhibition of direct oxidation of PRAS and the activation of its signaling pathway. Regulation of oxidized pPRAS after ischemic injury may provide a molecular target for therapeutic intervention.

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