**Oxidative Stress Affects the Integrin-Linked Kinase Signaling Pathway After Transient Focal Cerebral Ischemia**

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**Background and Purpose**—The integrin-linked kinase (ILK) signaling pathway contributes to regulation of cellular adhesion, migration, and differentiation, and to apoptotic cell death after a variety of cell death stimuli. We have reported that overexpression of copper/zinc superoxide dismutase (SOD1) reduces apoptotic cell death by promoting the phosphatidylinositol 3-kinase (PI3-K)/Akt survival pathway after transient focal cerebral ischemia (tFCI). However, the role of the ILK pathway after tFCI and the role of oxygen free radicals in regulation of apoptosis remain unclear.

**Methods**—To clarify these issues, we used an in vivo tFCI model with SOD1 transgenic mice and wild-type mice. We administered the PI3-K inhibitor, LY294002, into mouse brains after tFCI and examined the role of PI3-K in the ILK pathway and expression of the ILK/Akt complex by immunohistochemistry, Western blot analysis, and coimmunoprecipitation.

**Results**—A transient increase in ILK was detected early after tFCI and was prevented by treatment with LY294002, but promoted by SOD1. Coimmunoprecipitation revealed that the direct reaction of ILK/Akt transiently increased concurrent with the increase in ILK after tFCI. Moreover, the ILK/Akt complex was prevented by LY294002, but promoted by SOD1.

**Conclusions**—These results suggest that the ILK pathway mediated by PI3-K is affected by tFCI and by SOD1. (*Stroke*. 2004;35:2560-2565.)

**Key Words:** apoptosis ■ cerebral ischemia ■ superoxide dismutase

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The cell survival pathway has been the focus for clarifying the apoptotic neuronal cell death mechanisms. Integrin-linked kinase (ILK) is a novel apoptotic regulator that plays an important role in the Akt cell signaling pathway as a phosphatidylinositol-3 kinase (PI3-K)-dependent serine–threonine kinase. ILK regulates a variety of cellular reactions, including adhesion, migration, differentiation, and survival, via its pleckstrin homology-like domain and protein kinase catalytic domain. ILK is downstream from growth factors, such as nerve growth factor, and from integrin receptor activation. Stimulation of ILK results in activation of Akt and inhibition of glycogen synthase kinase-3.

The PI3-K–dependent Akt pathway is involved in apoptotic neuronal cell death and is downstream of both nerve growth factor and oxidative stress. Phosphorylated Akt transiently increases dependent on PI3-K after reperfusion injury after in vivo cerebral ischemia, and its activation is promoted by administration of nerve growth factor after stroke. Moreover, Akt substrates such as Bad and the proline-rich Akt substrate play a critical role in regulating apoptosis after cerebral ischemia. ILK is thought to function upstream of Akt to control cell survival in vitro and is thought to be involved in regulation of apoptotic neuronal cell death. However, the machinery of ILK remains unknown in vivo.

Reactive oxygen species (ROS) have been implicated in the machinery of reperfusion injury after cerebral ischemia. The electron flow in isolated brain mitochondria produces superoxide, which is scavenged by superoxide dismutase. We have shown that copper/zinc superoxide dismutase (SOD1), a cytosolic antioxidant isoenzyme, is highly protective against ischemia and reperfusion injury after cerebral ischemia. In recent studies, SOD1 overexpression promoted the key factors controlling cell survival such as Akt, phosphorylated Bad, and phosphorylated proline-rich Akt substrate after cerebral ischemia. The present study was designed to clarify the relationship of ILK to ROS after cerebral ischemia with the use of a model of reperfusion injury after transient focal cerebral ischemia (tFCI).

**Materials and Methods**

**SOD1 Transgenic Mice**
Heterozygous SOD1 transgenic (Tg) mice of the SOD1 TGHS/SF-218-3 strain (Neurosurgical Laboratories, Stanford University, Calif)
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. There were no differences in the phenotypes or in the regional cerebral blood flow before or after FCI between the SOD1 Tg mice and their wild-type littermates.

Focal Cerebral Ischemia

Adult male mice (3 months old, 35 to 40 grams) were subjected to tFCI by intraluminal middle cerebral artery blockade with a 6-0 nylon suture as described previously. 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.

Drug Treatment

LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Cell Signaling Technology] was dissolved in dimethyl sulfoxide and phosphate-buffered saline (PBS) (LY294002, 50 nmol in 25% dimethyl sulfoxide in PBS; vehicle, 25% dimethyl sulfoxide in PBS) and injected intraventricularly as described previously.

Immunohistochemistry

The brains were fixed in 4% paraformaldehyde and sectioned at 50 µm on a vibratome. The sections were incubated with blocking solution and reacted with rabbit polyclonal anti-ILK antibody (Upstate Cell Signaling Solutions, Charlottesville, Va) at a dilution of 1:400. Immunohistochemistry was performed using the avidin–biotin technique, and then the nuclei were counterstained with methyl green.

Western Blot Analysis

Protein extraction and immunoprecipitation were performed as described previously. Equal amounts of the samples were loaded per lane. The primary antibodies were 1:600 dilution of the antibody against ILK (Upstate Cell Signaling Solutions) or 1:10 000 dilution of anti-β-actin monoclonal antibody (Sigma Chemical Co, St Louis, Mo). 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. Two hundred micrograms of the protein sample were incubated with protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) and with an anti-ILK antibody (Upstate Cell Signaling Solutions) for 3 hours at 4°C. The pellets were used as the samples and were immunoblotted with an anti-Akt antibody (Cell Signaling Technology, Beverly, Mass) as described in the Western blot analysis.

In Situ Detection of Superoxide Anion Production

The early production of superoxide anions in cerebral ischemia was investigated using oxidized hydroethidine (HEt) as previously described. HEt solution (200 µM; 1 mg/mL in PBS) was administered intravenously 15 minutes before induction of ischemia. The brains were sectioned at 50 µm on a vibratome and were subsequently incubated with the anti-ILK antibody (Upstate Cell Signaling Solutions) as mentioned and with avidin-conjugated fluorescein isothiocyanate and 4',6 diamidino-2-phenylindole. They were then observed with a fluorescent microscope.

Quantification and Statistical Analysis

The data are expressed as mean±SD. We performed a 1-way analysis of variance followed by Fisher protected least significant difference test for multiple group analysis (SigmaStat software; Jandel Corporation). Comparisons between 2 groups were achieved using Student t test. Significance was accepted with \( P<0.05 \).

Results

ILK Expression Transiently Increased After tFCI

ILK protein immunopositive cells were seen in the cortex and the caudate putamen (Figure 1A). They were large and triangular and had round, large nuclei (Figure 1A). ILK was slightly expressed under nonischemic conditions and transiently increased 1, 2, and 4 hours after tFCI. ILK and β-actin from the cytosolic fraction samples in the nonischemic control brains (sham) and ischemic brains (lanes 1 hour to 24 hours) were analyzed with horseradish peroxidase-conjugated immunoglobulin G (Cell Signaling Technology) with the use of enhanced chemiluminescence detection reagents (Amersham International).

Figure 1

A, Representative photomicrographs show immunohistochemical staining for ILK in the nonischemic lesion (left) and in the ischemic lesion (right) 2 hours after tFCI (n=3). ILK protein immunopositive cells were seen in the cortex and the caudate putamen. The immunopositive cells were large and triangular and had round, large nuclei. ILK expression was strongly observed in the ischemic cortical region 2 hours after tFCI compared with the nonischemic region. Bar=50 µm. B, Western blot analysis of ILK and β-actin after tFCI. ILK and β-actin from the cytosolic fraction samples in the nonischemic control brains (sham) and ischemic brains (lanes 1 hour to 24 hours) were analyzed with horseradish peroxidase-conjugated immunoglobulin G (Cell Signaling Technology) with the use of enhanced chemiluminescence detection reagents (Amersham International).

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Coimmunoprecipitation Demonstrated the Direct Binding of ILK to Akt

In the cytosolic fraction, a band of ILK precipitated by the Akt protein (ILK/Akt) was detected in the sham control and transiently increased 2 hours after reperfusion (Figure 2). These results suggest that ILK binds directly to Akt and that the expression of this complex transiently increased concomitant with the transient increase of ILK after tFCI.

Inhibition of PI3-K Prevented the Expression of ILK and the ILK/Akt Complex After tFCI

Western blot analysis demonstrated that ILK significantly decreased in the LY294002-treated mice compared with the vehicle-treated mice 2 hours after tFCI (Figure 3A, *P<0.05). Coimmunoprecipitation demonstrated that expression of the ILK/Akt complex was also significantly decreased in the LY294002-treated mice compared with the vehicle-treated mice 2 hours after tFCI (Figure 3B; *P<0.05). There was no significant difference in ILK or ILK/Akt expression in the sham-operated samples between the LY294002-treated mice and the vehicle-treated mice (Figure 3A and 3B).

Major Superoxide Production Delocalized With the Increase in ILK Expression After tFCI

We analyzed in situ superoxide production by oxidized HET and compared the spatial expression of ILK with that of superoxide 2 hours after tFCI (Figure 4). The most oxidized HET expression did not colocalize with the strong expression of ILK. Oxidized HET was remarkably decreased and ILK immunoreactivity was strongly observed in the SOD1 Tg mice compared with the wild-type mice 2 hours after tFCI (Figure 4A and 4B).

Overexpression of SOD1 Increased Expression of ILK and the ILK/Akt Complex After tFCI

Western blot analysis demonstrated that ILK immunoreactivity was detected as a significantly stronger band in the SOD1 Tg mice compared with the wild-type mice 2 hours after reperfusion (Figure 5A; *P<0.05). Coimmunoprecipitation demonstrated that the expression of ILK/Akt significantly increased in the SOD1 Tg mice compared with the wild-type mice 2 hours after reperfusion (Figure 5B; *P<0.05). There was no significant difference in ILK or ILK/Akt expression in the sham-operated samples between the SOD1 Tg mice and the wild-type mice (Figure 5A and 5B).

Discussion

There is clear evidence that neuronal apoptotic cell death involves factors that determine the balance between cell death.
and survival. In recent studies, cell survival factors have been the focus as critical regulators of the progress of apoptosis. These include serine–threonine kinase, the Akt cell signaling pathway, mitogen-activated protein kinase, and extracellular signal-regulated kinase (ERK), and the ERK pathway. As a key regulator upstream of Akt and downstream of PI3-K, ILK has an important role via phosphorylation in response to different stimuli. The β1 subunit of the integrin receptor, which is the most promiscuous subunit of the ILK receptor, is widely expressed in the hippocampus, cortex, and brain stem and mediates signaling events critical to cell survival via ILK activation. The β1 subunit is involved in the activation of Akt phosphorylation and it can promote Akt phosphorylation on serine 473. ILK is activated after integrin stimulation, and dominant-negative ILK blocks integrin-mediated Akt activation and cell survival in hippocampal neuronal cells in vitro. However, little is known about the relationship between Akt and the ILK signaling pathway in vivo. In the present study, we demonstrated the following points: (1) expression of ILK transiently increased during the early period of reperfusion injury after tFCI; (2) the ILK/Akt complex was detected and transiently increased after tFCI; (3) inhibition of PI3-K prevented the transient increase of both ILK and ILK/Akt after tFCI; (4) oxidized HEt did not colocalize with the remarkable expression of ILK after tFCI; and (5) overexpression of SOD1 promoted the expression of both ILK and ILK/Akt after tFCI. As with the reported in vitro studies, ILK activation by PI3-K and the ILK/Akt complex were detected in our in vivo tFCI model. Akt expression did not change, but phosphorylated Akt (serine 473) transiently increased during the early periods of reperfusion injury after tFCI in our model. ILK is one of the activators of Akt, the activation of which may also be controlled by PI3-K. There is a possibility that Akt activation...
might be regulated by both the ILK signaling pathway and PI3-K. The upper band of ILK was affected more by PI3-K inhibition and SOD1, suggesting that this band may be involved in the phosphorylation of ILK. Further studies are needed to clarify the role of the ILK pathway in PI3-K activation after apoptotic stimuli.

We have demonstrated that there is an important link between the ILK pathway and superoxide after cerebral ischemia. Our data suggest that ILK activation is not associated with superoxide production after reperfusion injury and that SOD1 overexpression increased ILK because of superoxide inhibition after tFCI. Our earlier studies demonstrated that overexpression of intrinsic SOD1 protects against ischemic damage.9,14,16,17 The production and receptor-mediated activation of transforming growth factor (TGF) play important roles in cell survival after apoptotic stimuli.31,32 ROS affect TGF activation or production by reducing oxidation-sensitive transcription factors such as NF-xB and hypoxia-inducible factor-1 after apoptotic stimuli.33,34 TGF has recently been reported to promote ILK activation via the PI3-K signaling pathway to increase cell growth.35 TGF might link the interaction between ROS and the ILK signaling pathway after apoptotic stimuli. Little is known about the direct trigger for inducing ILK expression or the direct neuroprotective role of ILK. However, in the neuronal cells, which produced superoxide after reperfusion injury, ILK expression was reduced by the superoxide and was promoted by overexpression of SOD1. Further study is required to clarify the link between the ILK signaling pathway and oxidative stress after tFCI.

Conclusion

Our results imply that the ILK cell signaling pathway is activated in neurons after tFCI and that SOD1 contributes to the inhibition of apoptosis induced by FCI by reducing the early formation of superoxide radicals and promoting ILK. We suggest that ILK may contribute in part to the neuroprotective role of SOD1 after cerebral ischemia.

Acknowledgments

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References


28. Agius E, Sagot Y, Duprat AM, Cochard P. Antibodies directed against the \( \beta_1 \)-integrin subunit and peptides containing the IKVAV sequence of laminin perturb neurite outgrowth of peripheral neurons on immature spinal cord substrata. Neuroscience. 1996;71:773–786.


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