Oxidative Stress Is Associated With XIAP and Smac/DIABLO Signaling Pathways in Mouse Brains After Transient Focal Cerebral Ischemia

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Background and Purpose—The interaction of X chromosome-linked inhibitor-of-apoptosis protein (XIAP) with second mitochondria-derived activator of caspase (Smac)/direct inhibitor-of-apoptosis protein-binding protein with low pi (DIABLO) contributes to regulation of apoptosis after a variety of cell death stimuli, and in our reported in vivo transient focal cerebral ischemia (tFCI) model. We have also reported 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 XIAP signaling pathway and oxidative stress in the regulation of apoptosis after tFCI.

Methods—We used a tFCI model of SOD1 transgenic mice and wild-type littermates to examine the expression of XIAP and Smac/DIABLO by Western blotting and the interaction of XIAP with Smac/DIABLO (XIAP/Smac) or caspase-9 (XIAP/caspase-9) by coimmunoprecipitation. The direct oxidation of 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—Direct oxidative injury to cytosolic and mitochondrial proteins was reduced by SOD1 after tFCI. The individual oxidized carbonyls in XIAP, mitochondrial Smac/DIABLO, and caspase-9 were also reduced by SOD1. Expression of XIAP and XIAP/caspase-9 was promoted, whereas translocation of Smac/DIABLO and XIAP/Smac was reduced, by SOD1 after tFCI.

Conclusions—These results suggest that overexpression of SOD1 may affect the XIAP pathway after tFCI by reducing the direct oxidative reaction to XIAP regulators after reperfusion injury. (Stroke. 2004;35:1443-1448.)

Key Words: cerebral ischemia • superoxide dismutase • apoptosis

The apoptotic signaling pathway is regulated by a variety of factors and is based on the balance between cell death and survival factors. The central players in apoptosis are the caspases, and one important route to caspase activation involves the translocation of cytochrome c from mitochondria to the cytosol. An apoptosome forms when released cytochrome c interacts with Apaf-1 and caspase-9, both of which play essential roles in the mitochondrial apoptotic pathway. Second mitochondria-derived activator of caspases (Smac)/direct inhibitor-of-apoptosis protein (IAP)-binding protein with low pi (DIABLO) is released from mitochondria into the cytosol concurrently with cytochrome c and eliminates the inhibitory effects of IAP.

The IAP family includes cIAP1/2, X chromosome-linked IAP (XIAP), NIAP, and survivin. These proteins bind to and inhibit both initiator caspases such as caspase-9 and effector caspases. All IAPs contain baculovirus IAP repeat (BIR) domains. Among the IAPs, XIAP is the most potent inhibitor of caspases. The third BIR domain of XIAP in particular inhibits cleaved caspase-9. We have shown that the interaction of XIAP with Smac/DIABLO and the caspases plays a critical role in the regulation of apoptotic neuronal cell death after in vivo cerebral ischemia.

Reactive oxygen species have been implicated in the mechanism of reperfusion injury after cerebral ischemia. The electron flow in isolated brain mitochondria produces superoxide anions, which are scavenged by superoxide dismutase (SOD). We have shown that copper/zinc-SOD (SOD1), a cytosolic isoenzyme, is highly protective against ischemia and reperfusion injury after ischemia. Our studies have demonstrated that SOD1 affected the early release of cytochrome c and Smac/DIABLO from mitochondria in ischemia models in vivo. However, whether SOD1 affects IAP reaction remains unknown. The present study was designed to clarify the role of SOD1 in the XIAP pathway after transient focal cerebral ischemia (tFCI).
Focal Cerebral Ischemia

Adult male mice (3 months old, 35 to 40 g) were subjected to tFCI by intraluminal middle cerebral artery blockade with a 6-0 nylon suture as described previously.16,18 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.16 Equal amounts of the samples were loaded per lane. The primary antibodies were used previously.15,20 The mice were anesthetized with 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.

Coimmunoprecipitation

Protein extraction and immunoprecipitation were performed as described previously.24,25 Two hundred micrograms of the protein sample were incubated with protein G-Sepharose (Amersham Biosciences) and with an anti-XIAP antibody (BD Transduction Laboratories) for 3 hours at 4°C. The pellets were used as the samples and were immunblotted with anti-Smac/DIABLO antibody (Chemicon) and anticaspase-9 antibody (Santa Cruz Biotechnology) as described in the Western blot method.

Detection of Oxidized Carboxyls in Protein

A commercial detection assay was used with 2,4-dinitrophenylhydrazone (DNP) to examine the direct oxidative injury to proteins (Chemicon). The manufacturer’s methodological protocols were followed. DNP specifically reacted with the oxidized carboxyl residues on the protein samples.26 The DNP binding sites of the oxidized proteins were specifically detected using an anti-DNP antibody by Western blot or coimmunoprecipitation.26,27 We analyzed the density of each lane by the total expression of DNP.

Quantification and Statistical Analysis

The data are expressed as mean ± SD. We performed 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’s t test. Significance was accepted with P < 0.05.

Results

Effect of SOD1 Overexpression on the XIAP Pathway

In our previous study, XIAP transiently increased 8 hours after tFCI and Smac/DIABLO translocated from mitochondria into the cytosol 24 hours after tFCI.11 The direct binding of XIAP to both Smac/DIABLO (XIAP/Smac) and caspase-9 (XIAP/caspase-9) significantly increased 8 hours after tFCI.11 In the present study, we have now demonstrated that XIAP immunoreactivity was stronger in the SOD1 Tg mice than in the wild-type mice 8 hours after reperfusion (Figure 1A; *P < 0.05). Translocated cytosolic Smac/DIABLO (MW=25 kDa) significantly decreased in the SOD1 Tg mice compared with the wild-type mice 24 hours after tFCI (Figure 1B; *P < 0.05). Coimmunoprecipitation revealed that XIAP/Smac (MW=25 kDa) was significantly reduced in the SOD1 Tg mice compared with the wild-type mice 8 hours after reperfusion (Figure 1C; *P < 0.05). XIAP/caspase-9 (MW=15 kDa) significantly increased in the SOD1 Tg mice compared with the wild-type mice 8 hours after reperfusion (Figure 1D; *P < 0.05). There was no significant difference in the nonischemic controls (cont) between the wild-type and SOD1 Tg mice. Beta-actin was used as an internal control. OD indicates optical density; wt, wild-type.

Figure 1. Western blotting demonstrated that XIAP immunoreactivity (molecular weight [MW] = 57 kDa) was stronger in the SOD1 Tg mice than in the wild-type mice 8 hours after reperfusion (A) (*P < 0.05). Translocated cytosolic Smac/DIABLO (MW=25 kDa) significantly decreased in the SOD1 Tg mice compared with the wild-type mice 24 hours after tFCI (B) (*P < 0.05). Coimmunoprecipitation revealed that XIAP/Smac (MW=25 kDa) was significantly reduced in the SOD1 Tg mice compared with the wild-type mice 8 hours after reperfusion (C) (*P < 0.05). XIAP/caspase-9 (MW=15 kDa) significantly increased in the SOD1 Tg mice compared with the wild-type mice 8 hours after reperfusion (D) (*P < 0.05). There was no significant difference in the nonischemic controls (cont) between the wild-type and SOD1 Tg mice. Beta-actin was used as an internal control. OD indicates optical density; wt, wild-type.
50.5% ± 34.3%; *P < 0.05). XIAP/Smac was significantly reduced in the SOD1 Tg mice compared with the wild-type mice 8 hours after reperfusion (Figure 1C; n = 4, 68.6% ± 28.8%; *P < 0.05). XIAP/caspase-9 significantly increased in the SOD1 Tg mice compared with the wild-type mice 8 hours after reperfusion (Figure 1D; n = 4, 70.5% ± 41.3%; *P < 0.05).

Direct Oxidative Injury to Cytosolic and Mitochondrial Proteins After tFCI

We used Western blot analysis of DNP to examine oxidative injury to cytosolic and mitochondrial proteins by detecting the DNP binding sites. Total oxidative injury to cytosolic carbonyl proteins was reduced in the SOD1 Tg mice compared with the wild-type mice 1, 4, and 24 hours after tFCI (Figure 2A; 1 hour: n = 4, 28.1% ± 13.7%, *P < 0.05; 4 hours: n = 4, 28.9% ± 9.6%, *P < 0.05; 24 hours: n = 4, 40.2% ± 9.3%, *P < 0.05). Total oxidative injury to mitochondrial proteins was reduced in the SOD1 Tg mice compared with the wild-type mice 1, 4, and 24 hours after tFCI (Figure 2B; 1 hour: n = 4, 31.2% ± 8.2%, *P < 0.05; 4 hours: n = 4, 32.9% ± 12.1%, *P < 0.05; 24 hours: n = 4, 59.0% ± 6.0%, *P < 0.05).

Direct Oxidative Injury to the Individual Regulators of the XIAP/Smac Pathway

We examined oxidative injury to the individual proteins, indicated by oxidation of the carbonyl groups, with the use of samples precipitated by the DNP antibody after DNP binding. XIAP expression in the proteins bound to DNP (XIAP/DNP) remarkably increased 1 and 2 hours after tFCI (Figure 3A and B; n = 4, *P < 0.05). Smac/DIABLO in the DNP-bound proteins (Smac/DNP) was not detected in the cytosolic proteins after tFCI (data not shown); however, in the mitochondrial fraction, Smac/DNP was detected and increased 1 and 2 hours after tFCI (Figure 3A and B; n = 4, *P < 0.05). Caspase-9 in the DNP-bound proteins (caspase-9/DNP) significantly increased 8 hours after tFCI (Figure 3A and B; n = 4, *P < 0.05).

SOD1 Overexpression Reduced Oxidative Injury to the Individual Binding Proteins of the XIAP Pathway

Cytosolic XIAP/DNP (n = 4, 63.7% ± 15.4%, *P < 0.05) and mitochondrial Smac/DNP (n = 4, 73.8% ± 15.5%, *P < 0.05) were both significantly reduced in the SOD1 Tg mice compared with the wild-type mice 1 hour after tFCI (Figure 4A and B). Caspase-9/DNP was also reduced in the SOD1 Tg mice compared with the wild-type mice 8 hours after tFCI (Figure 4C; n = 4, 75.5% ± 32.5%, *P < 0.05).

Discussion

IAPs are potent regulators of cell death and cell survival after a variety of apoptotic stimuli.9,12 XIAP interacts with other protein regulators via dimerization, and BIR domains play a critical role in protein binding.6,12 In the cytosol, Smac/DIABLO binds BIR domains of XIAP to eliminate any inhibitory effect and other BIR domains of XIAP bind the initiator or the effector of caspase to inhibit them in many apoptotic models.8,9,28 In our previous study, we demonstrated that the interaction among XIAP, Smac/DIABLO, and caspases plays a critical role in regulation of apoptotic neuronal cell death after tFCI.11 However, how oxidative stress affects the XIAP pathway after reperfusion injury remains unknown. In the present study, we demonstrated the following points for the first time: (1) SOD1 overexpression increased XIAP and reduced translocated Smac/DIABLO after tFCI; and (2) SOD1 overexpression reduced binding of XIAP to Smac/DIABLO and increased the interaction of SOD1 Oxidative Stress Affects XIAP/Smac After Stroke

Figure 2. Western blot analysis revealed that oxidative injury to the total cytosolic proteins was reduced in the SOD1 Tg mice compared with the wild-type mice 1, 4, and 24 hours after tFCI (A) (*P < 0.05). The total oxidative injury to mitochondrial proteins was reduced in the SOD1 Tg mice compared with the wild-type mice 1, 4, and 24 hours after tFCI (B) (*P < 0.05). Fifty micrograms of protein for the cytosolic samples and 20 μg of protein for the mitochondrial samples were used. The densitometry of each smear band was calculated and quantitatively examined. Cont indicates control.
XIAP with caspase-9 after tFCI. We used methods for detecting DNP-binding sites on proteins to examine the targets of oxidative injury in the XIAP pathway and to find where SOD1 could protect against oxidative injury to the XIAP pathway. With the DNP assays, we demonstrated the following: (1) direct oxidative injuries to the cytosolic and mitochondrial proteins increased after tFCI, but were reduced by SOD1 overexpression; and (2) formation of oxidized carbonyls in XIAP, mitochondrial Smac/DIABLO, and caspase-9 was detected during the early period of reperfusion injury and these oxidized carbonyl proteins were all significantly reduced by SOD1 overexpression after tFCI. The precise physiological mechanisms of superoxide interaction with the carbonyl residues of those individual proteins remain unclear. Our results, however, suggest that overexpression of SOD1 might inhibit oxidative injury to neuronal cells at the level of intracellular proteins after in vivo tFCI.

Our previous studies demonstrated that SOD1 overexpression has protective effects against ischemic damage. In this study, we examined the role of SOD1 in the XIAP pathway after tFCI by focusing on the formation of oxidized carbonyls in the individual proteins. Superoxide production was detected 1 hour after reperfusion in our model. The total cytosolic and mitochondrial oxidized carbonyl proteins were shown as smear bands, the expression of which began to increase concurrently with superoxide production, which matches results of our previous report. Each band changed in a variety of ways, some being time-dependent and others not. These results suggest that total oxidation of proteins may accumulate in a time-dependent manner; however, oxidative stress may not injure each protein uniformly and the injuries may progress at various time points. Apoptotic cell death induced by oxidative injury is known to proceed to chain reactions. Our results suggest that not only an upstream substrate but also individual downstream substrates might be directly injured by oxidative stress and that each individual protein injured by oxidative stress might be rescued by SOD. Protein oxidation was quantitatively analyzed by measuring...
the amount of DNP, and it caused an enzymatic dysfunction of glutamine synthetase after ischemia/reperfusion injury in vivo.29 Oxidative stress caused structural changes in intracellular proteins as well as membranous lipids after reperfusion injury.30 The significant difference in protein expression between the SOD1 Tg mice and the wild-type mice in our study was in accord with the results of oxidized carboxyl protein expression by DNP assay. These results suggest that superoxide may directly or indirectly cause protein oxidation and that it may also affect the binding of proteins to other proteins.

Neuronal mitochondria are a major source of superoxide, and excessive superoxide production after tFCI may cause mitochondrial oxidative injury that leads to the release of cytochrome c from the intermembrane space.22 Accumulation of cytosolic Smac/DIABLO and release of cytochrome c may be controlled by continuous mitochondrial oxidative stress after reperfusion injury.10 In our study, Smac/ DIABLO had less oxidative injury in the cytoplasm, but more in mitochondria. This result suggests that mitochondrial oxidative injury may play an important role in the accumulation and the translocation of Smac/DIABLO after reperfusion. Overexpression of cytosolic SOD could reduce both cytosolic and mitochondrial oxidative injury. There are reports that SOD1 may exist in mitochondrial space as well in cytosolic space.31,32 Recent experiments revealed that voltage-dependent anion channels control the balance of superoxide between the mitochondrial and cytosolic space, and that overproduction of superoxide in the mitochondrial space spreads into the cytoplasmic space.33 This suggests that cytosolic SOD1 can contribute to the reduction of overproduced superoxide, not only in the cytosol but also in the mitochondrial space.33 However, the precise mechanism remains unknown and further studies are needed to clarify the machinery of SOD in mitochondrial injury.

In conclusion, SOD1 contributes to the inhibition of direct oxidation of carboxyls on binding-protein regulators of the XIAP pathway. The sensitivity of XIAP to oxidation during ischemia and reperfusion may provide a molecular target for therapeutic intervention.

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