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Akt/Bad signaling and motor neuron survival after spinal cord injury

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The serine–threonine kinase Akt is a cell survival signaling pathway that inactivates the proapoptotic BCL-2 family protein Bad and promotes cell survival in cerebral ischemia. Involvement of the Akt/Bad signaling pathway after spinal cord injury (SCI) is, however, uncertain. Our results showed that phospho-Akt (serine-473) and phospho-Bad (serine-136) were significantly upregulated at 1 day after SCI. In addition, phospho-Akt and phospho-Bad were colocalized in motor neurons that survived SCI and inhibition of PI3-K reduced expression of phospho-Akt and phospho-Bad. Dimerization of Bad with 14-3-3 in the cytosol was increased whereas Bad/Bcl-X_L binding in the mitochondria was decreased after SCI. We further found that reduced oxidative stress by SOD1 overexpression in rats enhanced the expression of phospho-Akt, phospho-Bad, Bad/14-3-3 binding and reduced Bad/Bcl-X_L binding after SCI, as compared to wild-type rats. We conclude that oxidative stress may play a role in modulating Akt/Bad signaling and subsequent motor neuron survival after SCI.

Keywords: Spinal cord injury; Akt; Bad; Apoptosis; SOD1; Oxidative stress

Introduction

The serine–threonine kinase Akt (protein kinase B) plays an important role in the cell death/survival pathway (Franke et al., 1997). Growth factors such as insulin-like growth factor-1 and platelet-derived growth factor activate Akt and promote cell survival through the phosphoinositide 3-kinase (PI3-K) pathway, which can be inhibited by wortmannin or LY294002 (Alessi et al., 1996; Datta et al., 1997). Akt is activated through phosphorylation at threonine-308 or serine-473 (Alessi et al., 1996, 1997). After phosphorylation, Akt functions through phosphorylation and inhibition of Bad (serine-136) (Blume-Jensen et al., 1998; Datta et al., 1997), glycogen synthase kinase-3 (Cross et al., 1995), forkhead transcription factors (Brunet et al., 2001), or caspase-9 (Cardone et al., 1998).

Bad is a proapoptotic member of the Bcl family that promotes cell death by dimerization with Bcl-2 or Bcl-X_L (Yang et al., 1995). Bad phosphorylation at four different serine residues (serine-112, -136, -155, or -170) has been characterized as inactivating Bad (Datta et al., 2000; Dramsi et al., 2002; Lizcano et al., 2000; Zha et al., 1996). After phosphorylation, Bad binds with the ubiquitous protein 14-3-3 in the cytosol and reduces the binding availability of unphosphorylated Bad to Bcl-X_L (Datta et al., 1997; Zha et al., 1996), thus promoting cell survival by freeing Bcl-X_L, a well-characterized anti-apoptotic member of the Bcl family.

Apoptosis plays an important role in neuronal loss after central nervous system injury. Studies have shown that oligodendrocytes, neurons, and glia undergo apoptosis (Crowe et al., 1997; Liu et al., 1997; Yong et al., 1998) and that caspase cascades such as caspase-3 and caspase-9, and cytochrome *c* and Bad are involved in apoptosis after spinal cord injury (SCI) (Springer et al., 1999, 2000; Sugawara et al., 2002). A temporal increase in Akt phosphorylation was reported after cerebral ischemia (Noshita et al., 2001; Ouyang et al., 1999) and traumatic brain injury (TBI) (Noshita et al., 2002) and this increase has been proposed to be neuroprotective. Reactive oxygen species play important roles in the pathogenesis of central nervous system injury. Human copper/zinc-superoxide dismutase (SOD1) is a crucial endogenous enzyme responsible for eliminating superoxide, and overexpression of SOD1 in transgenic (Tg) rats or mice reduces superoxide production and protects neurons from death after focal cerebral ischemia (Yang et al., 1994), global ischemia (Chan et al., 1998), and SCI (Sugawara et al., 2002). However, the involvement of Akt, and its downstream protein Bad in cell death/survival and oxidative stress after SCI is not clear. In this study, we used SOD1 Tg rats to study the relationship of Akt phosphorylation, Bad phosphorylation, and dimerization with 14-3-3 and Bcl-X_L.

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Materials and methods

SOD1 Tg rats

Heterozygous SOD1 Tg rats with a Sprague–Dawley background, carrying human SOD1 genes, were derived from the founder stock and further bred with wild-type (Wt) Sprague–Dawley rats to generate heterozygous rats as previously described by our group (Chan et al., 1998). The phenotype of the SOD1 Tg rats was identified by isoelectric focusing gel electrophoresis as described (Chan et al., 1998). There were no observable phenotypic differences in brain vasculature between the Tg rats and Wt littermates (Chan et al., 1998).

Surgery

All animals were treated in accordance with Stanford University guidelines and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Adult female SOD1 Tg rats (250–300 g) and their Wt littermates were used in this experiment. Animals were anesthetized with 2.0% isoflurane in 70% N_2O and 30% O_2 using a face mask. Lumbar enlargement was exposed by a partial laminectomy of the T13 vertebra, and a vascular clip (closing force 15 *g*; Ohwa Tsusho, Tokyo, Japan) was applied extradurally for 5 s according to our previous study (Sugawara et al., 2002). The rectal temperature was controlled at 37.0 ± 0.5°C during surgery with a homeothermic blanket.

Drug injection

To investigate the role of the PI3-K pathway after SCI, we administered a PI3-K inhibitor, LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) (Cell Signaling Technology, Beverly, MA, USA), as previously described (Noshita et al., 2001). LY294002 was dissolved in dimethyl sulfoxide to make a stock solution of 100 mM, and further diluted to 25 mM in filtered phosphate-buffered saline (PBS, pH 7.4) before use. A partial laminectomy was performed and lumbar enlargement was exposed. Two microliters of 25 mM LY294002 and the same amount of the vehicle (25% dimethyl sulfoxide in PBS) were injected intrathecally 2 h before SCI.

Immunohistochemistry

Anesthetized animals were perfused with 10 U/ml heparin saline and subsequently with 3.7% formaldehyde in PBS 1, 3, and 7 days after SCI. Spinal cords at the lumbar enlargement were collected and postfixed with the same fixative for 24 h. The tissue was embedded in paraffin and sectioned 6 μm thick with a microtome. We focused on two different areas, the core area from the epicenter to 3 mm rostral and 3 mm caudal, and the peripheral area 3–6 mm rostral to the epicenter and caudal counterpart. For immunohistochemistry, sections were deparaffinized and incubated with 3% H_2O_2 in PBS. We used a 5% blocking serum, and then incubated the sections with rabbit polyclonal anti-phospho-Akt (serine-473) antibody (1:50; Cell Signaling Technology) at 4°C overnight. The sections were then reacted with biotin-conjugated goat anti-rabbit immunoglobulin G (1:100; Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h, then incubated with avidin–biotin horseradish peroxidase solution (ABC kit; Vector Laboratories) for 30 min at room temperature and visualized using 0.025% 3,3′-diaminobenzidine hydrochloride (DAB kit; Vector Laboratories). Finally, the sections were counterstained with methyl green and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Double staining

To investigate the colocalization of phospho-Akt (serine-473) with phospho-Bad (serine-136), double staining of phospho-Bad with phospho-Akt was performed. After deparaffinization, the sections were blocked in 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and incubated with monoclonal mouse anti-phospho-Akt antibody (1:50; Cell Signaling Technology) at 4°C overnight. Then, the sections were reacted with Texas-Red-conjugated donkey anti-mouse immunoglobulin G (1:100; Jackson ImmunoResearch) at room temperature for 1 h. The sections were incubated with polyclonal rabbit anti-phospho-Bad antibody (1:50; Oncogene Research Products, San Diego, CA, USA) at 4°C overnight followed by incubation with fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (1:100; Jackson ImmunoResearch). Finally, the sections were mounted on slides using VECTASHIELD (Vector Laboratories) and visualized with a fluorescent microscope. For control immunostaining, we either deleted the primary antibody or used an excessive amount of antigen for antibody absorption prior to the immunostaining procedure.

Western blot analysis

To obtain the whole cell fraction, spinal cord from the peripheral area was collected 1, 3, and 7 days after SCI and gently homogenized by douncing 30 times in a glass tissue grinder (Wheaton, Millville, NJ, USA) in 5 volumes of cold suspension buffer (20 mM HEPES-KOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 tablet of cocktail inhibitor [Roche Diagnostics, Mannheim, Germany] in 25 ml buffer). The homogenates were centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was collected and protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Approximately 30 μg of total protein per lane were subjected to sodium dodecyl sulfate-polyacrylamide-gel electrophoresis on a Tris–glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred to a polyvinylidene difluoride membrane (Invitrogen). The membrane was incubated in polyclonal rabbit anti-phospho-Akt (serine-473) antibody (1:1000; Cell Signaling Technology), polyclonal rabbit anti-phospho-Bad (serine-136) antibody (1:1000; Oncogene Research Products), monoclonal mouse anti-14-3-3 antibody (1:2000; Chemicon International, Temecula, CA, USA), or polyclonal rabbit anti-Bcl-X_L antibody (1:2000; Cell Signaling Technology) overnight at 4°C, and then incubated in the peroxidase-conjugated secondary antibody. The signals were detected with a chemiluminescence kit (Amersham International, Buckinghamshire, UK) and exposed on X-ray film. To confirm equal loading, the membranes were then stained with β-actin. After the film was scanned with a GS-700 imaging densitometer (Bio-Rad), we performed a quantitative analysis using Multi-Analyst software (Bio-Rad). Optical density of each band was measured on the same membrane ($n \geq 4$), the average value of the controls was calculated and taken as 1 (100%) and relative values were calculated using optical density of each band divided by average value of the controls. A statistical study was performed using these relative values and the results are presented as mean ± SEM.