JOURNAL OF NEUROTRAUMA 25:1121–1131 (September 2008)

DOI: 10.1089/neu.2008.0544

Estrogen-Induced Bcl-2 Expression after Spinal Cord Injury Is Mediated through Phosphoinositide-3-Kinase/Akt-Dependent CREB Activation

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Abstract

Our previous study showed that, after spinal cord injury (SCI) in rats, estrogen provides neuroprotection through expression of Bcl-2. However, molecular targets that mediate estrogen-induced expression of Bcl-2 are not fully understood. Here, we investigated whether, after SCI, the phosphatidylinositol-3 kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) pathways are involved in estrogen-induced expression of Bcl-2. Both Akt and ERK were activated and peaked at 8 h after SCI. Treatment with estrogen significantly increased the level of phosphorylated Akt (pAkt) and ERK (pERK) after injury. Cyclic-AMP response element binding protein (CREB) transcription factor was also activated and peaked at 8 h after SCI. Treatment with estrogen significantly increased the level of phosphorylated CREB (pCREB) after injury. Administration of LY294002, an inhibitor of PI3K/Akt, decreased the level of pCREB after SCI, whereas PD98059, an inhibitor of ERK, showed no significant effect. Also, treatment with LY294002 significantly inhibited expression of Bcl-2, but PD98059 showed no significant effect. Furthermore, treatment with estrogen inhibited apoptotic cell death, whereas treatment with LY294002 or PD98059 increased apoptotic cell death after SCI. Together, these data indicate that estrogen's neuroprotection is mediated in part by induction of Bcl-2 through PI3K/Akt-dependent CREB activation.

Key words: Akt; Bcl-2; CREB; estradiol; spinal cord injury

Introduction

ESTROGEN HAS BEEN SHOWN to delay the onset of Alzheimer's disease (Henderson et al., 1996; Tang et al., 1996; Kawas et al., 1997), reduce the incidence and mortality of stroke patients (Paganini-Hill, 1995), and protect neurons in animal models of brain ischemia (Chen et al., 1998; Dubal et al., 1998; Wang et al., 1999; Horsburgh et al., 2002; Jover et al., 2002; Bagetta et al., 2004). Furthermore, we previously showed that systemic administration of estrogen prevents neuronal death and thus improves the functional recovery after spinal cord injury (SCI) (Yune et al., 2004). These observations indicate that estrogen protects neurons against a variety of central nervous system (CNS) disorders and injuries.

Estrogen has been shown to activate extracellular signal-regulated kinase (ERK) that mediates neuroprotection *in vitro* and *in vivo* (Singer et al., 1999; Kuroki et al., 2001; Jover-Mengual et al., 2007). Also, treatment with estrogen induces the activation of Akt in the hypothalamus *in vivo* (Cardona-Gomez et al., 2002) and inhibits hydrogen peroxide—induced retinal neuronal cell death through the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway *in vitro* (Yu et al., 2004). Together, these results indicate that estrogen's neuroprotection is mediated through both Akt and ERK pathways *in vivo* and *in vitro*.

Up-regulation of the expression of Bcl-2 by estrogen has been identified as a critical mechanism promoting cell survival after brain injury (Dubal et al., 1999; Soustiel et al., 2005), transient middle cerebral artery occlusion (MCAO) (Alkayed et al., 2001), and SCI (Yune et al., 2004). Also, cyclic-AMP response element binding protein (CREB) transcription factor has been identified as a positive regulator of expression of Bcl-2 (Wilson et al., 1996; Pugazhenthi et al., 1999, 2000; Freeland et al., 2001). Akt is known to activate CREB (Du and Montminy 1998), which plays an important role in regulating cell survival and death (Dudek et al., 1997). ERK can also trigger CREB activation through its phosphoryla-

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tion (Xing et al., 1998). Thus, it is reasonable to postulate that Akt and ERK activation leading to CREB activation may mediate estrogen-induced expression of Bcl-2 after SCI. Whether both the Akt and ERK signaling cascades are involved in estrogen-induced expression of Bcl-2 after SCI, however, has not been determined.

In the present study, we examined the role of PI3K/Akt and ERK activation in estrogen-induced expression of Bcl-2 and in apoptotic cell death after SCI. In addition, we determined the involvement of CREB transcription factor in a signaling mechanism for estrogen-induced expression of Bcl-2 after injury. Our data showed that, after SCI, neuroprotection by estrogen was mediated in part by expression of Bcl-2 through PI3K/Akt dependent-CREB phosphorylation, although another pathway mediated by estrogen-induced ERK activation might also be involved in cell survival after injury.

Methods

Materials

T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). CREB consensus oligonucleotide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Estrogen (17β-estradiol; 2-hydroxypropyl-β-cyclodextrin [β-cyclodextrin]-encapsulated), nuclear extract preparation kit, and other chemicals used in this study were purchased from Sigma (St. Louis, MO).

Spinal cord injury

Adult rats (Sprague-Dawley; male; 250—300 g; Sam:TacN [SD] BR; Samtako, Osan, Korea) were anesthetized with chloral hydrate (500 mg/kg), and a laminectomy was performed at the T9-T10 level, exposing the cord beneath without disrupting the dura. The exposed dorsal surface of the cord was subjected to contusion injury (10 g $\times$ 12.5 mm) using an NYU impactor as previously described (Lee et al., 2003; Yune et al., 2004). For the sham-operated controls, the animals underwent a T9-T10 laminectomy without weight-drop injury. All surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committee of the Kyung Hee University.

Drug administration

Estrogen was dissolved in sterile 0.1 M phosphate-buffered saline (PBS), pH 7.2, and rats received intravenous injections of β-cyclodextrin-encapsulated estrogen (100 μg/kg) or β-cyclodextrin solution 1 h after SCI (n = 3/group for molecular work; n = 4/group for TUNEL). Our previous report showed that 100 mg/kg of estrogen was an optimal dose for functional recovery after SCI (Yune et al., 2004). Intraspinal injection of LY294002 (Calbiochem, La Jolla, CA), an inhibitor of PI3K/Akt, or PD98059 (Calbiochem), an inhibitor of ERK, was performed as previously described (n = 3 or 4/group) (Lee et al., 2000; Yune et al., 2003). In brief, 2 μL of LY294002 (10 or 50 nmol) or PD98059 (30 or 100 μmol), dissolved in 10% or 50% dimethyl sulfoxide (DMSO), respectively, was injected into the epicenter of the lesion at 1 h after SCI. Control groups received injections of equal volumes of 10% or 50% DMSO at the corresponding time points.

Tissue preparation

Animals were anesthetized with chloral hydrate (500 mg/kg) and perfused via cardiac puncture initially with 0.1 M PBS (pH 7.4) and subsequently with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Spinal cord tissue (20 mm) centered at the lesion site was dissected out, post-fixed by immersion in the same fixative overnight, and placed in 30% sucrose in 0.1 M PBS (pH 7.4). The spinal tissue was embedded in OCT for frozen sections. Longitudinal or transverse sections were then cut at 10 or 20 μm on a cryostat (CM1850; Leica, Germany).

TUNEL and immunohistochemical staining

One day after injury, spinal cord tissues were prepared as described above (n = 4/group). Serial longitudinal sections were processed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining and then for immunohistochemistry using a neuron-specific nuclear protein (NeuN, 1:1000; Millipore, Billerica, MA) as previously described (Lee et al., 2003; Yune et al., 2004). Only double-labeled cells (TUNEL + NeuN) from each section were considered and counted as TUNEL-positive neurons in the gray matter (GM; from centromedial to ventral region). For quantification of TUNEL-positive neurons, serial transverse sections (10 μm thickness) were collected every 100 μm rostral and caudal to a distance of 3000 μm to the lesion epicenter (total 60 sections). The counting was performed by investigators who were blind as to the experimental conditions. Also, immunohistochemical staining was performed with polyclonal antibodies against phosphorylated Akt (pAkt; Ser473; Cell Signaling, Danvers, MA), phosphorylated ERK (pERK; Thr202/Tyr204; Cell Signaling), phosphorylated CREB (pCREB, 1:500; Cell Signaling), or Bcl-2 (1:500, Santa Cruz Biotechnology). Some sections labeled with pAkt, pERK, and pCREB antibodies were double-labeled with antibodies to NeuN (1:1000; Millipore), CC1 (1:200; Oncogene, Cambridge, MA), or Bcl-2 (1:500; Millipore). The sections were blocked in 5% normal serum and 0.1% Triton X-100 in PBS for 1 h at room temperature and then incubated with primary antibodies for overnight at 4°C , followed by horseradish peroxidase (HRP)—conjugated, FITC, and/or cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Also, nuclei were labeled with 4'-6-diamidino-2-phenylindole (DAPI) according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA). Images were collected using an Olympus microscope connected via CoolSNAP. In all immunohistochemistry controls, reaction to the substrate was absent if the primary antibody was omitted or if the primary antibody was replaced by a non-immune, control antibody.

Western blot

Rats were anesthetized 1, 4, 8, and 24 h after injury and perfused via cardiac puncture with 0.1 M PBS (pH 7.4) as described above (n = 3/group). Segments of spinal cord (10 mm) that centered upon the lesion site were isolated and homogenized in 1 mL of lysis buffer containing 1% NP-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride

in a Dounce homogenizer. Tissue homogenate was centrifuged at 25,000×*g* for 30 min at 4°C, and the protein levels of the supernatant were determined using the BCA assay (Pierce, Rockford, IL). Fifty micrograms of protein was separated by 10% sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore) by electrophoresis. The membranes were blocked with 5% nonfat skim milk in tris-buffered saline (TBS)/0.1% Tween 20 for 1 h at room temperature and then incubated with polyclonal antibodies against Bcl-2 (Santa Cruz Biotechnology), β-actin (Sigma), Akt, pAkt (Ser473, 60 kDa), ERK, pERK (Thr202/Tyr204; 44 and 42 kDa), CREB, and pCREB (1:1000, Cell Signaling) at 4°C overnight. The membranes were then processed with HRP-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). Immunoreactive bands were visualized by chemiluminescence using Supersignal (Pierce). Experiments were repeated three times to ensure reproducibility.

Electrophoretic mobility shift assay

The spinal cord tissues were isolated and processed as described above. Three spinal cords per group were pooled, and nuclear extracts were prepared using a CelLytic NuCLEAR Extraction kit (Sigma) according to the manufacturer's instructions. After quantification of protein, the nuclear extracts were used in an electrophoretic mobility shift assay (EMSA) by a method described previously (Huang et al., 2001; Yune et al., 2004). In brief, an oligonucleotide for cyclic-AMP response element (CRE) containing a consensus sequence (Santa Cruz Biotechnology) was radiolabeled with [^{32}P]-ATP (ICN, Costa Mesa, CA) by T4 polynucleotide kinase (New England Biolabs) to produce double-stranded DNA probes. Ten micrograms of nuclear protein were added to 20 μL of binding buffer (10 mM Tris-HCl, 20 mM NaCl, 1 mM DTT, and 1 mM EDTA, with 5% glycerol, pH 7.6) containing 1 μg of poly (dI-dC) and ^{32}P-labeled double-stranded DNA probe (100,000 cpm) and incubated at room temperature for 20 min. After incubation, the reaction mixture was subjected to electrophoresis on a nondenaturing 6% polyacrylamide gel at 180 V for 2 h under low ionic strength conditions. To visualize bands, the gel was dried and subjected to autoradiography. For competition experiments, radiolabeled DNA probes and nuclear proteins were incubated with a 100-fold molar excess of the unlabeled DNA oligonucleotide. Supershift assay was also performed by preincubating nuclear extracts with pCREB antibody (2 μg, Cell Signaling) for 1 h at 4°C before adding labeled probes. Relative intensity of each band on Western blots and EMSA was measured and analyzed by AlphaImager software (Alpha Innotech Corporation, San Leandro, CA). Background in films was subtracted from the optical density measurements. Experiments were repeated three times, and the values obtained for the relative intensity were subjected to statistical analysis.

Statistical analysis

Data are presented as mean ± standard deviation (SD) values. Quantitative data from TUNEL-positive cell counting and densitometric analyses of gels were evaluated for statistical significance using Student's paired *t*-test. In all analyses, a p value of <0.05 was considered statistically significant.

Results

Estrogen increases Akt and ERK activation after SCI

Since both Akt and ERK mediate protective effects of estrogen on neuronal survival *in vitro* and *in vivo* (Singer et al., 1999; Kuroki et al., 2001; Wilson et al., 2002; Yu et al., 2004; Jover-Mengual et al., 2007), we first examined whether Akt and ERK are activated after SCI. Western blotting was performed using antibodies against pAkt and pERK. As shown in Figure 1a, the level of pAkt was increased and peaked at 8 h after injury, but the level of total Akt was not changed up to 48 h post-injury (Fig. 1a). Quantitative analysis of Western blots revealed that the level of pAkt was increased 2.75-fold at 8 h after injury as compared to the sham control (Fig. 1a). The level of pERK was also increased and peaked at 8 h after injury (Fig. 1b). The level of total ERK was not changed up to 48 h after injury (Fig. 1b). Quantitative analysis of Western blots revealed that the level of pERK was increased 2.63-fold at 8 h after injury as compared to the sham control (Fig. 1b). We also examined the cell types expressing pAkt and pERK after SCI. Double-labeling using the cell type—specific markers, NeuN for neurons and CC1 for oligodendrocytes, revealed that the nuclei of neurons in the GM and oligodendrocytes in the white matter (WM) were positive for pAkt and pERK after injury (Fig. 1c). No pAkt- or pERK-positive cells were observed in the sham control (Fig. 1c). Next, we examined the effect of estrogen on Akt and ERK activation after injury. As shown in Figure 2, treatment with estrogen significantly increased the levels of pAkt and pERK when compared with those of vehicle control. Quantitative analysis of Western blots revealed that treatment with estrogen increased pAkt and pERK levels 2.1- and 1.6-fold, respectively, at 8 h after injury as compared with vehicle control (Fig. 2a,b). However, estrogen treatment did not change the levels of pAkt and pERK in uninjured spinal cord (data not shown).

Estrogen increases CREB activation after SCI

Since CREB transcription factor is known to act as a positive regulator of Bcl-2 induction (Wilson et al., 1996; Pugazhenthi et al., 1999, 2000; Freeland et al., 2001), we investigated whether CREB is activated following SCI. Western blotting was performed using a specific antibody for pCREB. As shown in Figure 3a, pCREB was increased up to 24 h after injury. We also examined the cell types expressing pCREB after SCI. Double-labeling revealed that the nuclei of neurons in the GM and oligodendrocytes in the WM were positive for pCREB (Fig. 3b). No pCREB immunoreactivity was observed in the sham control (Fig. 3b). Next, we performed EMSA to investigate the DNA binding activity of CREB after SCI. EMSA showed that DNA binding activity of CREB was increased and peaked at 1—8 h after injury although multiple DNA-protein complexes were observed (Fig. 3c). Since estrogen is known to activate CREB in the neuronal cells (Wu et al., 2005), we examined the effect of estrogen on the phosphorylation and DNA-binding activity of CREB after SCI. Treatment with estrogen significantly increased the level of pCREB at 8 h after injury when compared to that of vehicle control (Fig. 4a). Quantitative analysis of Western blots revealed that the treatment with estrogen increased pCREB 2.3-fold at 8 h after injury as com-

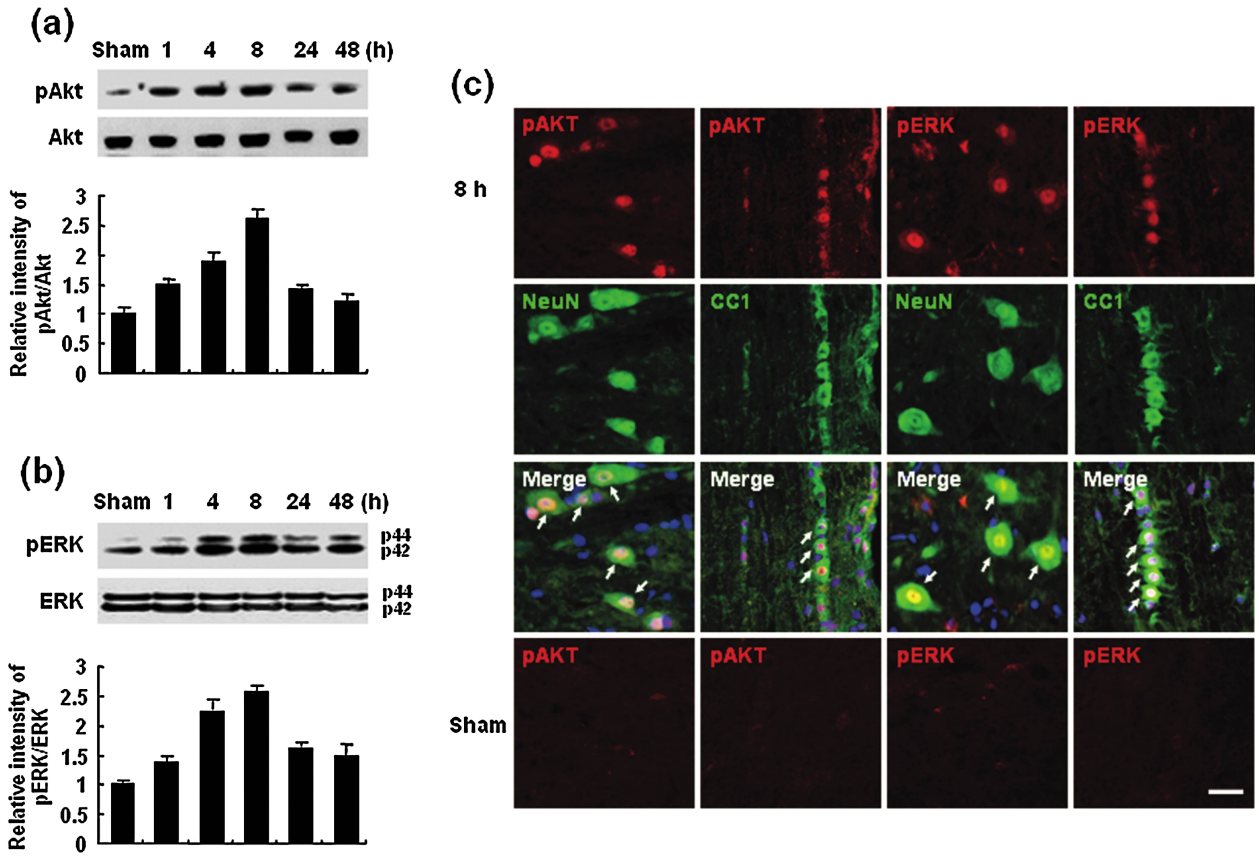

FIG. 1. Akt and ERK are activated after spinal cord injury (SCI). Spinal tissues and protein extracts were prepared at the time points indicated (1, 4, 8, 24, and 48 h after injury) as described in the Methods ($n = 3$/group). The gels presented are representative of results from three separate experiments. (**a**) Western blot of Akt and pAkt. Akt is shown as an internal control. Quantitative analysis revealed that the relative intensity of pAkt was increased 2.75-fold at 8 h after injury as compared to the sham control. (**b**) Western blot of ERK and pERK. Quantitative analysis revealed that the relative intensity of pERK was increased 2.63-fold at 8 h after injury as compared to the sham control. ERK is shown as an internal control. (**c**) Immunohistochemical analysis of pAkt and pERK. Double-labeling shows that neurons in the GM and oligodendrocytes in the WM were positive for both pAkt and pERK after SCI (arrows), while sham controls were negative for both pAkt and pERK. Representative photographs of longitudinal sections of 8 h post-injured tissues were taken from a location 3 mm rostral to the lesion site. Scale bar = 20 μm.

pared to that of vehicle control (Fig. 4a). Estrogen treatment did not change the level of pCREB in uninjured spinal cord (data not shown). DNA-binding activity of CRE was also significantly increased by treatment with estrogen at 8 h after injury (Fig. 4b). The specificity of the DNA-protein complexes was verified by supershift assay using CREB antibody. As shown in Figure 4b (lane 4), these complexes were supershifted by antibody against CREB. Quantitative analysis revealed that the treatment with estrogen increased the DNA-binding activity of CREB 1.8-fold as compared to that of vehicle control (Fig. 4c).

CREB phosphorylation is mediated through PI3K/Akt activation after SCI

Since estrogen is known to activate CREB transcription factor through the ERK and/or Akt signaling cascades in cultured hippocampal neurons (Wu et al. 2005; Lee et al., 2004) and MCAO in rat (Choi et al., 2004), we first examined whether LY294002, a PI3K/Akt inhibitor, and PD98059, an ERK inhibitor, would inhibit activation of PI3K/Akt and ERK, respectively, after SCI. After SCI, LY294002 (10 or 50 nmol) or PD98059 (30 or 100 μmol) was injected directly into the spinal cord at the lesion epicenter immediately after injury, and spinal cord tissues were prepared 8 h after injury. Treatment with either LY294002 or PD98059 significantly inhibited the levels of pAkt and pERK, respectively, as compared to vehicle controls (Fig. 5a,b). Next, we examined the effect of PI3K/Akt or ERK inhibitor on the CREB activation after SCI. As shown in Figure 5c, after injury, the phosphorylation of CREB was significantly inhibited by LY294002, but not by PD98059 indicating that CREB is activated through the PI3K/Akt signaling cascade.

Estrogen-induced Bcl-2 expression is mediated through PI3K/Akt activation

We have shown that estrogen enhances neuronal survival by increasing expression of Bcl-2 after SCI (Yune et al., 2004). To test whether ERK or Akt is involved in estrogen-induced expression of Bcl-2, we examined the effects of PI3K/Akt (LY294002) and ERK inhibitors (PD98059) on expression of

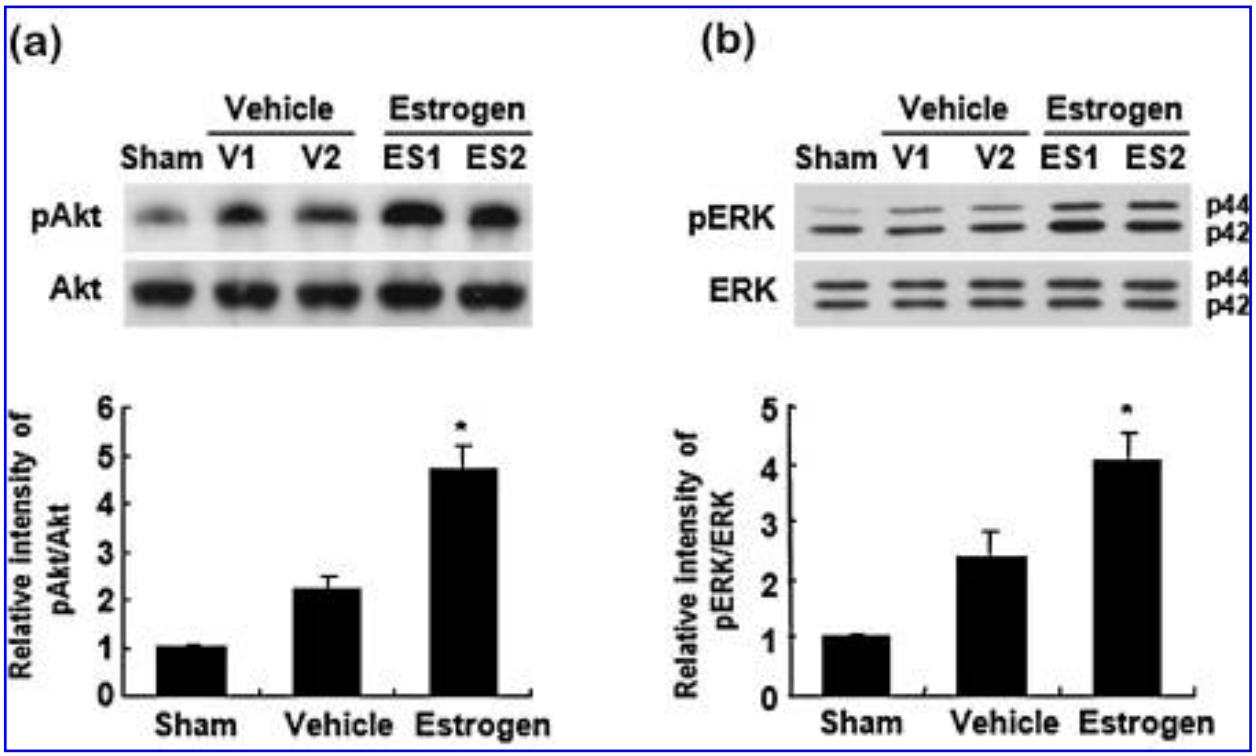

FIG. 2. Estrogen increases Akt and ERK activation after spinal cord injury (SCI). Spinal extracts were prepared 8 h after injury as described in Methods ($n = 3$/group). The gels presented are representative of results from three separate experiments. (**a**) Western blot of pAkt. Quantitative analysis of Western blots revealed that estrogen significantly increased the level of pAkt when compared to that in vehicle control. Values are means ± SD of three separate experiments. *$p < 0.001$. (**b**) Western blot of pERK. Quantitative analysis of Western blots revealed that treatment with estrogen significantly increased the level of pERK when compared to that in vehicle control. Values are means ± SD of three separate experiments. *$p < 0.001$.

Bcl-2 after SCI. As shown in Figure 6a, the treatment with estrogen significantly increased expression of Bcl-2 at 24 h after SCI. Estrogen-induced expression of Bcl-2 was significantly inhibited by LY294002 but not by PD98059 as compared to the control (Fig. 6a). These results indicate that estrogen-induced Bcl-2 expression is mediated through PI3K/Akt activation but not through ERK activation after injury. To examine whether estrogen-induced Bcl-2 is co-expressed with pCREB, which has been known to be activated by PI3K/Akt signaling (Du and Montminy, 1998), we performed double-labeling using antibodies against Bcl-2 and pCREB. Immunohistochemical analyses revealed that Bcl-2-positive neurons in the GM were positive for pCREB after injury (Fig. 6b), while pCREB-positive oligodendrocytes in the WM were negative for Bcl-2 (data not shown). These results suggest that estrogen-induced expression of Bcl-2 in neurons is mediated by PI3K/Akt-dependent CREB activation.

Inhibition of PI3K/Akt or ERK increases apoptotic cell death after SCI

PI3K/Akt and ERK are well-known signaling pathways that mediate cell survival and death (Singer et al., 1999; Kuroki et al., 2001; Wilson et al., 2002; Yu et al., 2004; Jover-Mengual et al., 2007). To investigate whether PI3K/Akt and ERK pathways are involved in cell survival or cell death after SCI, we injected an inhibitor of PI3K/Akt or ERK, LY294002 (10 nmol) or PD98059 (50 μmol), respectively, into the lesion after injury. The apoptotic cell death was then examined by TUNEL staining at 24 h after injury. TUNEL-positive neurons were observed primarily in the lesion area as previously reported (Liu et al., 1997; Yune et al., 2004) (Fig. 7a), while TUNEL-positive oligodendrocytes were not observed in the WM at 24 h after injury (data not shown). As shown in Figure 7b, estrogen treatment significantly reduced the number of TUNEL-positive neurons when compared to that of vehicle control as previously reported (Yune et al., 2004). Moreover, treatment with LY294002 or PD98059 significantly increased the number of TUNEL-positive cells when compared to vehicle control (Fig. 7b). The number of TUNEL-positive cells was increased by 33.8% or 28.6% after injury as a result of treatment with LY294002 or PD98059, respectively. These results indicate that both PI3K/Akt and ERK signaling pathways mediate cell survival after SCI.

Discussion

Our previous study showed that treatment with estrogen prevents neuronal cell death by inducing Bcl-2-expression after SCI (Yune et al., 2004). However, the mechanism by which estrogen induces Bcl-2-expression after injury has not been determined. The objective of the present study was to elucidate the molecular targets that mediated estrogen-induced Bcl-2-expression after SCI. Our results show that PI3K/Akt, ERK and CREB transcription factor were acti-

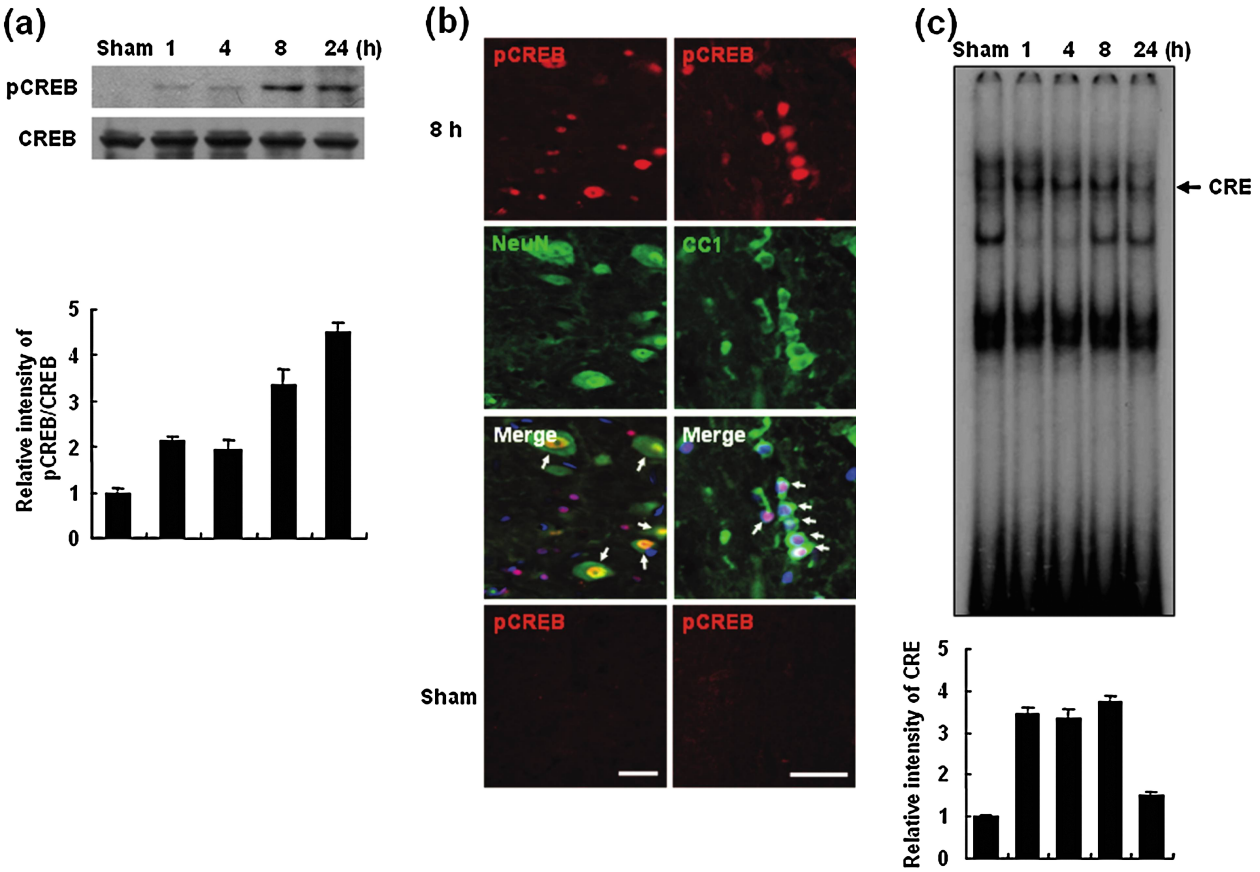

FIG. 3. CREB is activated after spinal cord injury (SCI). Spinal cord tissues were prepared at 1, 4, 8, and 24 h after SCI as described in the Methods ($n = 3$ for each time point). (**a**) Western blot of phosphorylated CREB (pCREB). CREB served as an internal control for protein loading. The gels presented are representative of results from three separate experiments. Quantitative analysis revealed that the relative intensity of pCREB was increased 4.5-fold at 24 h after injury as compared to that of sham control. Values are means ± SD of three separate experiments. (**b**) Immunohistochemical analysis of pCREB at 8 h after SCI. Arrows indicate the pCREB-positive nuclei of neurons in the GM and oligodendrocytes in the WM. Longitudinal sections of spinal cord tissues were taken from 3 mm rostral to the lesion site. Scale bars = 20 μm. (**c**) DNA-binding activity of CREB by EMSA using a 5′-end labeled consensus oligonucleotide. Note that multiple bands interacting with radiolabeled CRE DNA were observed in sham and injured spinal extracts. The protein complexes interacting with the CRE oligonucleotide increased at 1, 4, and 8 h after injury and decreased thereafter as compared to the sham control. The gels presented are representative of results from three separate experiments. Quantitative analysis revealed that the relative intensity of DNA-binding activity of CREB peaked at 1–8 h after injury as compared to that of sham control. Values are means ± SD of three separate experiments.

vated after SCI. Also, treatment with estrogen further increased activation of PI3K/Akt, ERK, and CREB after injury. Furthermore, CREB activation, which is involved in the estrogen-induced Bcl-2 expression after SCI, was mediated by the PI3K/Akt pathway. When considered together, our results suggest that, after SCI, estrogen provides neuroprotection through the PI3K/Akt/CREB/Bcl-2 signaling cascades.

The PI3K/Akt pathway plays a pivotal role in cell survival through several downstream targets, including the Bad, Forkhead transcription factors, and glycogen synthase kinase-3β (GSK3β) (Datta et al., 1997; Srinivasan et al., 2005; Endo et al., 2006). For example, Akt phosphorylates BAD and blocks the BAD-induced cell death of primary neurons (Datta et al., 1997). In addition, several studies showed a temporal increase in pAkt after cerebral ischemia (Ouyang et al., 1999; Noshita et al., 2001; Yano et al., 2001), brain injury (Noshita et al., 2002), and spinal ischemia (Sakurai et al., 2001, 2003), suggesting that the activation of PI3K/Akt pathway endogenously after various CNS injuries may serve as a "defense mechanism" by inactivating apoptotic proteins. Also, ERK is activated during various physiological and pathological events including ischemia and traumatic SCI (Yu and Yezierski, 2005; Crown et al., 2006; Jover-Mengual et al., 2007). These results are in agreement with our data that showed both Akt and ERK were activated after SCI. Furthermore, our results showed that an inhibitor of PI3K/Akt or ERK increased apoptotic cell death after injury; this suggests that both pathways are involved in cell survival after SCI. Also, estrogen activates both PI3K/Akt and ERK signaling transduction and reduces cell death induced by metabolic inhibition and hydrogen peroxide (Wilson et al., 2002; Yu et al., 2004). Estrogen reduces the neuronal cell death in ischemia *in vivo* and cerebral cortical explants *in vitro* (Dubal et al. 1998; Singer et al., 1999; Wang et al., 1999). In addition,

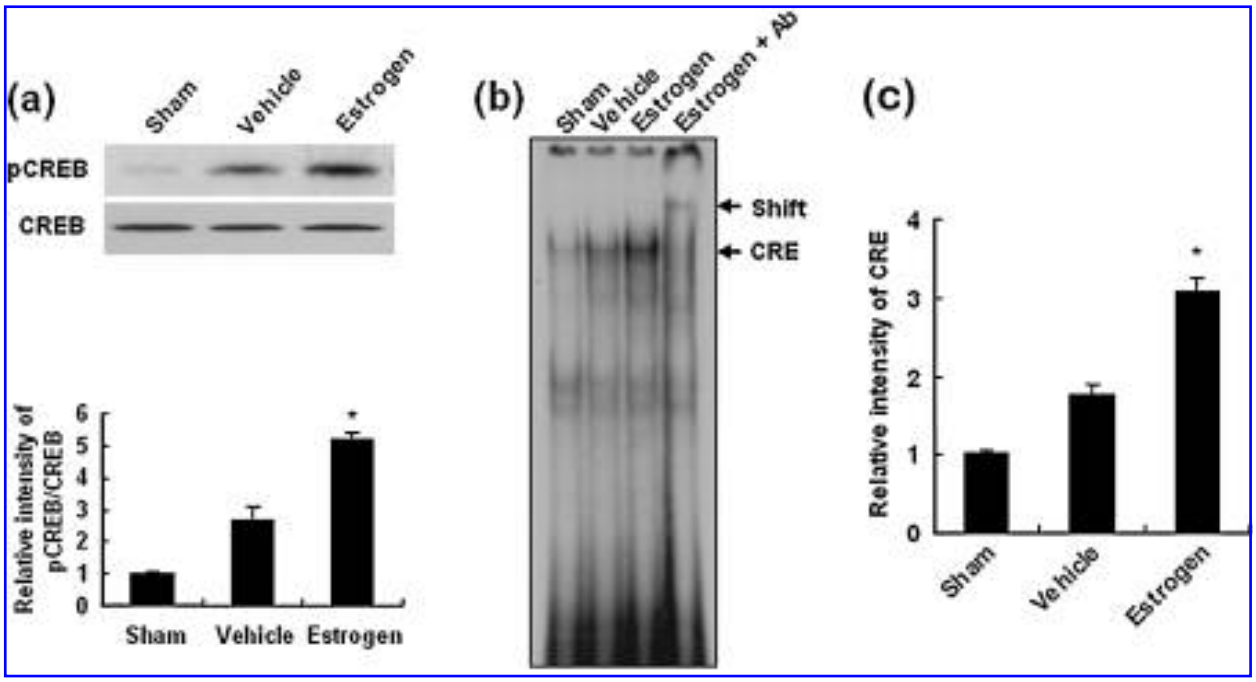

FIG. 4. Estrogen increases CREB activation after spinal cord injury (SCI). Total or nuclear proteins were prepared 8 h after injury as described in Methods ($n = 3$/group). (**a**) Western blot of pCREB. Quantitative analysis of Western blots revealed that estrogen significantly increased the level of pCREB when compared to that in vehicle control. Values are means ± SD of three separate experiments. *$p < 0.001$. (**b**) The DNA-binding activity of CREB by EMSA at 8 h after injury. The specificity of the DNA-protein complex was verified by incubation with CREB antibody. Gel shows that the DNA-protein complex was shifted by treatment with CREB antibody (see Estrogen + Ab). The gels presented are representative of results from three separate experiments. (**c**) Quantitative analysis revealed that DNA-binding activity of CREB was increased 1.8-fold by treatment with estrogen when compared to that of vehicle control. Values are means ± SD of three separate experiments. *$p < 0.001$.

our data show that estrogen further increases Akt and ERK activation, and reduces apoptotic cell death after SCI. When considered together, these observations suggest that an increase in cell survival by estrogen is likely to be mediated by both PI3K/Akt and ERK activation after CNS injuries. Furthermore, our results indicate that neuroprotection by estrogen after SCI is mediated by Bcl-2-expression through PI3K/Akt-dependent CREB activation. Typically, ERK is ac-

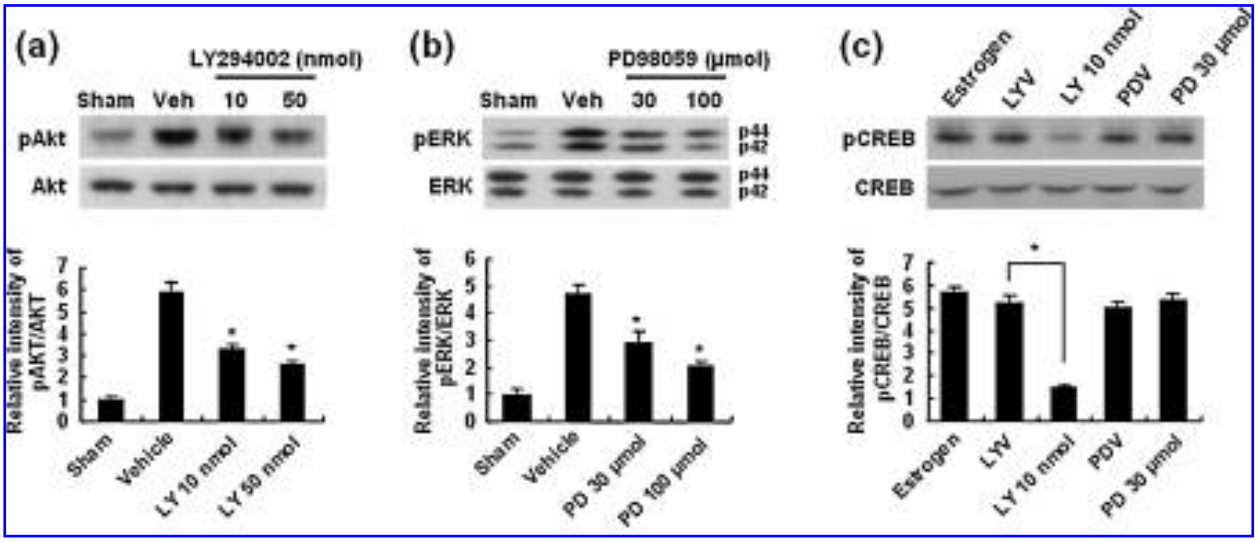

FIG. 5. Estrogen-induced CREB phosphorylation was dependent on PI3K/Akt activation. Spinal extracts were prepared at 8 h after injury as described in Methods ($n = 3$/group). (**a**) Western blot of pAkt. *$p < 0.001$. (**b**) Western blot of pERK. *$p < 0.001$. (**c**) Western blot of pCREB. Each densitometry reading of gel bands was expressed as an intensity relative to the injured control. Data represent means ± SD of three separate experiments. *$p < 0.001$.

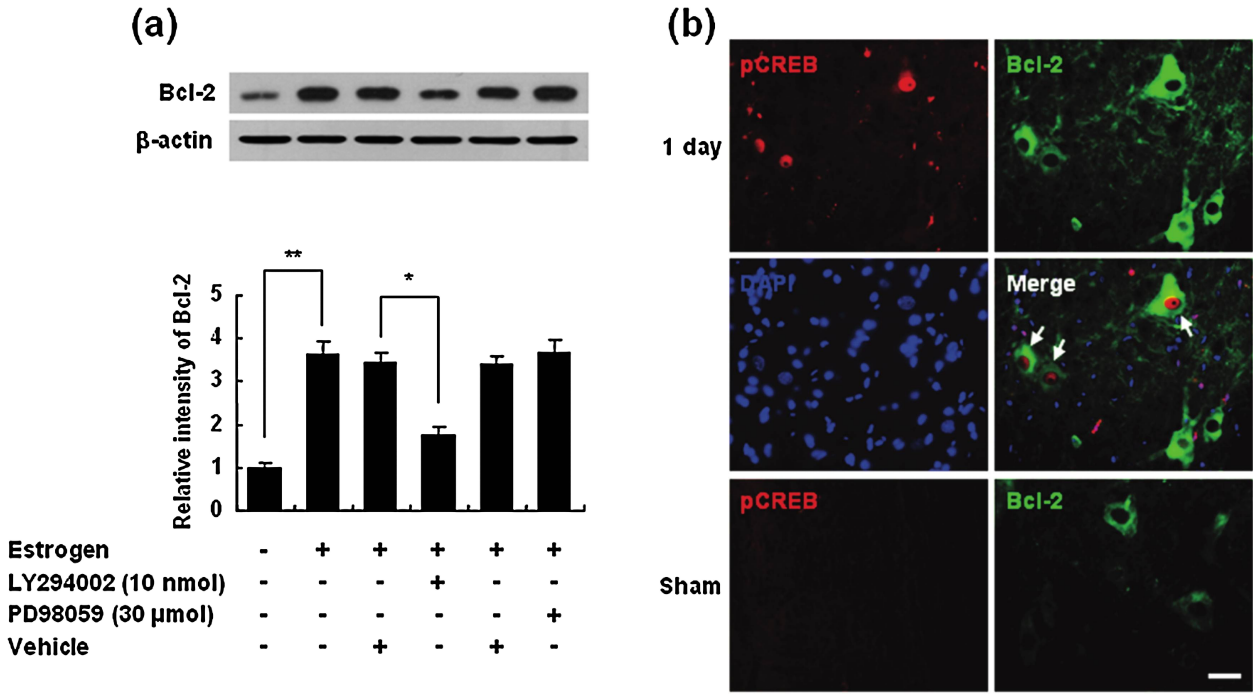

FIG. 6. Estrogen-induced Bcl-2 expression was dependent upon PI3K/Akt activation. Spinal cord extracts were prepared 24 h after injury as described in Methods ($n = 4$/group). (**a**) Western blot of Bcl-2 expression. The β-actin is shown as an internal control. Densitometry readings of gel bands were expressed as an intensity relative to the injured control. Data represent means ± SD of three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.001$. (**b**) Immunohistochemical analysis of Bcl-2 and pCREB after SCI. Double-labeling revealed that Bcl-2-positive neurons in the GM were positive for pCREB after injury (arrows); Bcl-2-positive cells showing low intensity were observed in sham control, but pCREB-positive cells were not observed. Longitudinal sections of spinal cord tissues at 24 h after injury were taken from rostral 3 mm to the lesion site. Scale bar = 20 μm.

tivated by signaling from receptor tyrosine kinase or G protein-coupled receptors (Neary, 2004). PI3K/Akt is also activated by such receptor tyrosine kinases as insulin-like growth factor I receptor (IGF-IR) and insulin receptor β-subunit (IRβ) (Rajala and Anderson 2001; Cardona-Gomez et al., 2002; Yu et al., 2004). However, whether estrogen activates PI3K/Akt and ERK after SCI through these upstream molecules was not examined in the present study.

Estrogen is known to act through genomic or non-genomic mechanisms. In the present study, we did not examine whether estrogen receptors themselves are involved in the neuroprotection by estrogen after SCI. Both estrogen receptors, α and β, are known to be expressed in neurons and glial cells of the spinal cord (Platania et al., 2003). Also, estrogen receptors may be involved in hormone-mediated neuroprotection after ischemic brain injury (Dubal et al., 1999). Moreover, a protein–protein interaction between estrogen receptor and the regulatory subunit p85 of PI3K leads to activation of both the Akt and MAPK signaling pathways in cortical neurons (Mannella and Brinton, 2006). Therefore, estrogen receptors are likely involved in promoting survival pathways in neurons after SCI.

CREB transcription factor is known to trigger the expression of survival genes such as Bcl-2 (Wilson et al., 1996; Xiang et al., 2006). CREB is also activated by phosphorylation via PI3K/Akt and ERK signaling transduction (Choi et al., 2004; Lee et al., 2004; Wu et al., 2005). Furthermore, CREB-dependent gene expression has been implicated in complex and diverse processes within the nervous system, including development, plasticity, and disease (Lonze and Ginty 2002). Our results showed that CREB was activated after SCI, and its activation was further increased by treatment with estrogen. Our study using inhibitors of PI3K/Akt and ERK also showed that CREB activation by treatment with estrogen was dependent upon PI3K/Akt activation after SCI. Since CREB activation is known to be involved in the induction of Bcl-2-expression by hypoxia in neuronal cells (Freeland et al., 2001), our data suggest that estrogen-induced Bcl-2-expression is likely mediated through PI3K/Akt/CREB signaling cascades and that neuroprotection mediated by estrogen after SCI appears to be mediated in part by Bcl-2-expression.

The target genes of CREB are diverse and categorized within groups that contain consensus CRE sequence-molecules related to neurotransmission, growth factors, and hormones, and molecules related to cellular metabolism, transcription factors, and signal transduction (Lonze and Ginty 2002). Promoter analysis of the bcl-2 gene reveals that it contains CRE in the 5′ regulatory region that binds CREB and related family members; the mutation of CRE blocks stimulus-induced bcl-2 transcription (Wilson et al., 1996), suggesting that the transcription of bcl-2 by CREB activation is a way that other extracellular signals including estrogen might promote cell survival. Our results indicate that estrogen treatment activates CREB, which is mediated through PI3K/Akt activation after injury. Furthermore, our immunohistochemical observations revealed that Bcl-2-positive

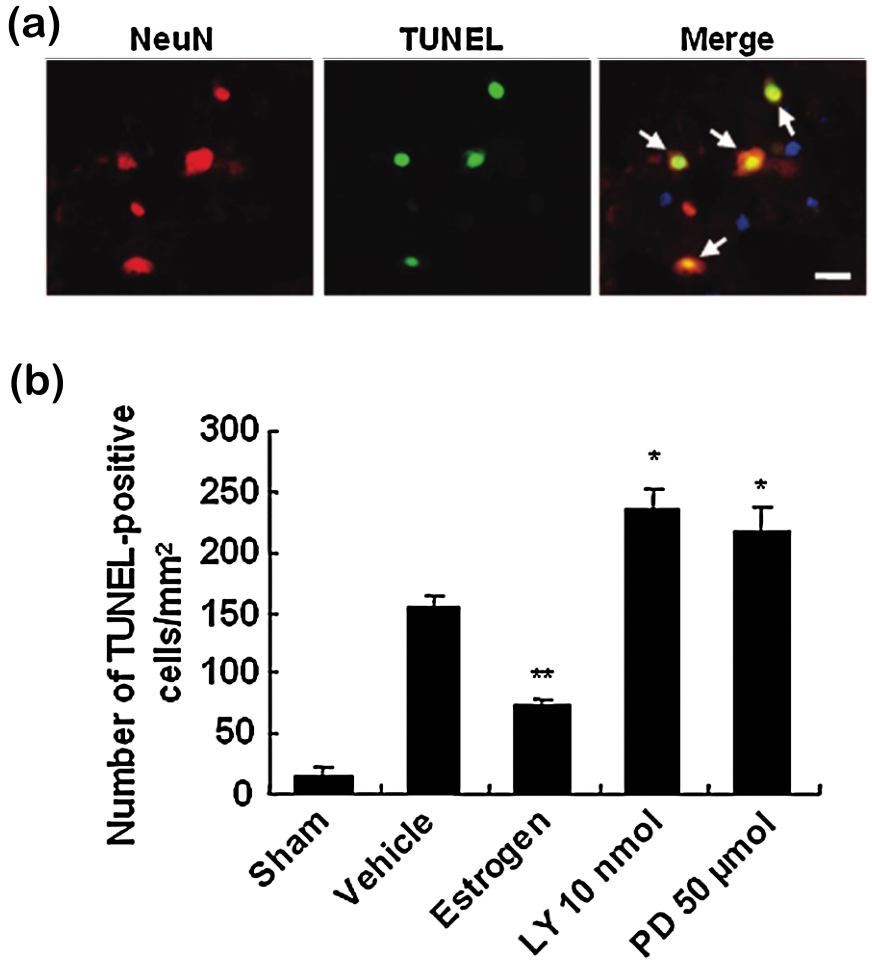

FIG. 7. Inhibition of Akt or ERK increases apoptotic cell death after SCI. Spinal cord tissues were collected 24 h after injury (n = 4/group). Serial longitudinal sections were processed for double-labeling (TUNEL + NeuN) as described in Methods. (**a**) Representative photographs of TUNEL (green)—positive and NeuN (red)—positive cells (arrows) in injured spinal tissue 24 h after SCI were taken from a location 2 mm rostral to the lesion site. Scale bar = 30 μm. (**b**) Quantification of the number of double (TUNEL + NeuN)—positive cells. Only double-labeled cells were considered and counted as TUNEL-positive neurons in the GM (from centromedial to ventral region) extending from 3 mm rostral to 3 mm caudal to the lesion epicenter from each section. Data represent means ± SD of three separate experiments. *$p < 0.05$, **$p < 0.001$.

cells were colocalized with pCREB after SCI, further suggesting that PI3K/Akt/CREB cascade is likely involved in estrogen-induced Bcl-2-expression after injury.

Estrogen has been shown to exert a neuroprotective effect mediated by the SRC/ERK/CREB/Bcl-2 signaling cascade in cultured hippocampal neurons (Wu et al., 2005). A recent report shows that the PI3K/Akt signaling pathway is involved in neuroprotection by estrogen in hydrogen peroxide-induced apoptosis of rat retinal neurons (Yu et al., 2004). Our data showed that inhibition of either the PI3K/Akt or ERK pathway results in an increase in the number of apoptotic cells after SCI. Also, our previous report showed that estrogen administration reduces neuronal cell death by inducing anti-apoptotic Bcl-2-expression after SCI (Yune et al., 2004). Taken together, these results suggest that estrogen protects neurons after SCI from insults mediated through both PI3K/Akt and ERK signaling pathways. Nevertheless, our results indicate that the neuroprotective effect of estrogen is mediated, at least partially, by Bcl-2-expression through PI3K/Akt/CREB signaling pathway after SCI. That being said, other pathways mediated by estrogen-induced ERK activation are also likely involved in neuronal survival after injury.

A recent report (Swartz et al., 2007) showed that estrogen has no neuroprotective effect in either male or female rats after SCI. However, other reports (Furlan et al., 2005; Farooque et al., 2006) showed that mouse or human females have better outcomes following SCI than males. Our previous report (Yune et al., 2004) and the present study show that estrogen has neuroprotective effects in male rats after SCI. In experimental stroke models, estrogen treatments reduced brain injury in both male and female rats (Toung et al., 1998; Rusa et al., 1999). Therefore, the discrepancy in response to estrogen in males versus females observed in these studies may be attributable to the degree of injury, estrogen level, and/or experimental model.

In conclusion, activation of PI3K/Akt and ERK after SCI mediate estrogen-induced activation of CREB transcription

factor, increasing anti-apoptotic Bcl-2 expression. This study provides further evidence that acute estrogen administration may be a beneficial therapeutic agent for acute SCI.

Acknowledgments

We wish to thank Dr. George Markelonis at the University of Maryland School of Medicine for his editorial assistance. This study was supported in part by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea Government (MOST) (no. R01-2007-000-20617-0, M1041200011-07N1200-01110), Seoul R & BD Program (no. 10524), and Post BK21 Program.

Author Disclosure Statement

No conflicting financial interests exist.

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