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Systemic Administration of 17β-Estradiol Reduces Apoptotic Cell Death and Improves Functional Recovery following Traumatic Spinal Cord Injury in Rats

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ABSTRACT

Recent evidence indicates that estrogen exerts neuroprotective effects in both brain injury and neurodegenerative diseases. We examined the protective effect of estrogen on functional recovery after spinal cord injury (SCI) in rats. 17β-estradiol (3, 100, or 300 μg/kg) was administered intravenously 1–2 h prior to injury (*pre-treatment*), and animals were then subjected to a mild, weight-drop spinal cord contusion injury. Estradiol treatment significantly improved hind limb motor function as determined by the Basso-Beattie-Bresnahan (BBB) locomotor open field behavioral rating test. Fifteen to 30 days after SCI, BBB scores were significantly higher in estradiol-treated (100 μg/kg) rats when compared to vehicle-treated rats. Morphological analysis showed that lesion sizes increased progressively in either vehicle-treated or 17β-estradiol-treated spinal cords. However, in response to treatment with 17β-estradiol, the lesion size was significantly reduced 18–28 days after SCI when compared to vehicle-treated controls. Terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) staining and DNA gel electrophoresis revealed that apoptotic cell death peaked 24–48 h after injury. Also, SCI induced a marked increase in activated caspase-3 in the spinal cord, evident by 4 h after injury. However, administration of 17β-estradiol significantly reduced the SCI-induced increase in apoptotic cell death and caspase-3 activity after SCI. Furthermore, 17β-estradiol significantly increased expression of the anti-apoptotic genes, bcl-2 and bcl-x, after SCI while expression of the pro-apoptotic genes, bad and bax, was not affected by drug treatment. Finally, intravenous administration of 17β-estradiol (100 μg/kg) immediately after injury (*post-treatment*) also significantly improved hind limb motor function 19–30 days after SCI compared to vehicle-treated controls. These data suggest that after SCI, 17β-estradiol treatment improved functional recovery in the injured rat, in part, by reducing apoptotic cell death.

Key words: apoptosis; BBB score; bcl-2; bcl-x; caspase-3; DNA laddering; estrogen; functional recovery; lesion area; neuroprotection; spinal cord injury; TUNEL

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INTRODUCTION

SPINAL CORD INJURY (SCI) is a devastating human experience from both the medical and psychological standpoints. SCI initiates a complex series of cellular and molecular events that induces the "programmed death" of neurons and glia thereby leading to permanent neurological deficits (Crowe et al., 1997; Emery et al., 1998; Li et al., 1996; Liu et al., 1997; Shuman et al., 1997; Yong et al., 1998). The development of any form of pharmacological therapy that can reduce or alleviate even some of the adverse outcomes associated with SCI has proven difficult due to the complexity of the injury. Furthermore, after SCI in humans, no therapeutic agent has proven effective in restoring motor functions. Accordingly, other pharmacological treatments must be explored for acute SCI that largely focus on attenuating secondary tissue damage.

Many studies have shown that estrogen treatment in postmenopausal women reduced the incidence of stroke and the extent of ischemic injury (Dubal et al., 1998; He et al., 2002; Pelligrino et al., 1998; Schmidt et al., 1996; Simpkins et al., 1997) and delayed the onset of neurodegenerative disorders, including Alzheimer's disease (Henderson et al., 1996; Kawas et al., 1997; Tang et al., 1996) and stroke (Paganini-Hill, 1995; Schmidt et al., 1996). Tamoxifen, an estrogen receptor antagonist, also increases the incidence of stroke in premenopausal women (Gail et al., 1999). Estrogen administration to ovariectomized female (Dubal et al., 1998; Dubal and Wise, 2001; Rusa et al., 1999) or to male (Toung et al., 1998) rats reduces brain injury after focal ischemia. Moreover, females with circulating estrogen are more resistant to focal ischemia than their male counterparts (Alkayed et al., 1998; Hall et al., 1991; Zhang et al., 1998). Estrogen exerts a neuroprotective effect in experimental models of neuronal injury. For example, estradiol treatment prevents a reduction in dopamine concentrations in the striatum of ovariectomized mice challenged with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (Callier et al., 2000; Disshon and Dluzen, 1997; Dulzen, 2000). Estrogen treatment in ovariectomized rats also prevents hilar neuronal loss in the dentate gyrus following kainic acid challenge (Azcoitia et al., 1998). In addition, estrogen attenuates neuronal injury caused by hypoxia, glutamate, NMDA, AMPA, superoxide anions, and hydrogen peroxide in primary neuronal cultures (Regan and Guo, 1997; Sawada et al., 1998; Singer et al., 1996; Weaver et al., 1997; Zaulyanov et al., 1999). In neuronal cell lines such as NT2, PC12, SK-N-SH, and HT 22, estrogen exerts neuroprotective effects against hydrogen peroxide-, glutamate-, serum deprivation-, lipid peroxidation- and β-amyloid-induced neurotoxicity (Goodman et al., 1996; Green et al., 1997; Singer et al., 1998; Vedder et al., 1999). These observations clearly indicate that estrogen provides neuroprotection against a variety of toxic insults to the CNS.

Bcl-2 is normally expressed at low levels in adult brain (Merry and Korsmeyer, 1997); its expression is influenced by the presence of estrogen in normal brain (Garcia-Segura et al., 1998). Expression of Bcl-2 is also induced in neurons after ischemic insults (Chen et al., 1997; Honkaniemi et al., 1996), resulting in increased cell viability (Chen et al., 1995; Shimazaki et al., 1994). In male animals, overexpression of Bcl-2 ameliorates cerebral ischemic injury (Antonawich et al., 1999; Kitagawa et al., 1998; Linnik et al., 1995; Martinou et al., 1994), whereas suppression of its expression by a gene deletion (Hata et al., 1999) or by treatment with an antisense oligonucleotide (Chen et al., 2000) exacerbates brain injury. Estrogen increases Bcl-2 expression but decreases Bax expression, resulting in the suppression of cyclical cell death in normal female reproductive tissues (Goodman et al., 1998; Sabourin et al., 1994). Furthermore, estrogen increases Bcl-2 expression which may account for the hormone-induced protection from glutamate- or hydrogen peroxide-induced toxicity in neuronal cells (Singer et al., 1998). These results thus suggest that the neuroprotective effects of estrogen may be mediated in part by regulating the expression of bcl-2, an anti-apoptotic gene.

Based on the results described above, we hypothesized that estrogen may also exert neuroprotective effects in SCI. This supposition has not been examined previously. In the present study, we examined the protective effects of 17β-estradiol on apoptotic cell death and functional recovery after SCI. We also examined whether estradiol modulates expression of the bcl-2 family of genes such as Bcl-2, Bcl-x, Bad, and Bax. Our results showed that estradiol treatment reduced apoptotic cell death and improved functional recovery after SCI. Furthermore, our data indicate that estradiol's neuroprotective effects may be mediated in part by modulating expression of bcl-2, an anti-apoptotic gene.

MATERIALS AND METHODS

Materials

17β-estradiol (Cyclodextrin-encapsulated) was purchased from Sigma (Sigma, St. Louis, MO). The Apoptag peroxidase kit for TUNEL-staining was purchased from Oncor (Oncor, Gaithersburg, MD). The monoclonal antibody against bcl-2 was purchased from Chemicon (Chemicon, Temecula, CA). Z-DEVD-AFC, a caspase-3 enzyme substrate and a polyclonal antibody against cas-

pase-3 were purchased from Enzyme System (Enzyme Systems Products, Livermore, CA) and Cell Signaling (Cell Signaling, Beverly, MA), respectively. Trizol Reagent and MMLV reverse transcriptase were purchased from GibcoBRL (GibcoBRL, Gaithersburg, MD).

Spinal Cord Injury (SCI)

Traumatic injury was induced using the calibrated weight drop device developed at New York University (Gruner, 1992) and followed a protocol developed by a multicenter consortium (Multicenter Animal Spinal Cord Injury Study; Basso et al., 1995, 1996 a,b), which has been reported previously (Liu et al., 1997; Xu et al., 1998). Briefly, adult [Sprague-Dawley; male; 250–300 g; Sam:TacN (SD) BR, Korea] rats were anesthetized with chloral hydrate (50 mg/kg, i.p.), and a laminectomy was performed at the T9–T10 level exposing the cord beneath without disrupting the dura. After the spinous processes of T8 and T11 were clamped to stabilize the spine, the exposed dorsal surface of the cord was subjected to a weight drop impact using a 10 gm rod (2.5 mm in diameter) dropped at a height of 12.5 mm. During surgery, rectal temperature was maintained at 37°C by a thermostatically regulated heating pad. After injury, the muscles and skin were closed in layers, and the rats were placed in a temperature and humidity-controlled chamber overnight. Manual expression of the urinary bladder was performed three times per day until reflex emptying was established. For the sham-operated controls, the animals underwent a T10 laminectomy without weight-drop injury. All surgical interventions and postoperative animal care were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committees of the Korea Institute of Science and Technology (KIST).

17β-Estradiol Administration

17β-Estradiol was dissolved in sterile 0.1 M PBS, pH 7.4 and administered intravenously 1–2 h prior to injury in rats (*pre-treatment*) randomly assigned by weight to control or to a treatment group (for behavioral testing, $n = 8$ for each vehicle or for estradiol, total 32 rats; for lesion area analysis, $n = 3$ for vehicle or estradiol, total 12 rats; for TUNEL staining, $n = 3$ for vehicle or estradiol, total 12 rats; for caspase-3 activity, $n = 3$ for sham-operated, vehicle or estradiol, total 9 rats; for bcl-2 expression, $n = 3$ for normal, sham-operated, vehicle or estradiol, total 39 rats). Rats received intravenous injections of 17β-estradiol (3, 100, and 300 μg/kg) or of vehicle, PBS saline (Shi et al., 1998; Toung et al., 1998; Yang et al., 2000). For the sham-operated controls, the animals underwent a T10 laminectomy without cord injury, received no pharmacological treatment and were sacrificed at the same time intervals as the treatment groups. In some experiments, rats receiving the 12.5-mm weight-drop insult received intravenous injections of 17β-estradiol at a dose of 100 μg/kg immediately after SCI (*post-treatment*) (for behavioral testing, $n = 8$ for each vehicle or for estradiol, total 16 rats).

Behavioral Test

The Basso-Beattie-Bresnahan (BBB) locomotor rating scale ranges from 0 to 21, where zero reflects no locomotor function and 21 reflects a normal performance; the rating was used to evaluate the effect of exogenous estrogen on functional recovery after SCI (Basso et al., 1995; $n = 8$ per group). Rats were allowed to walk around freely in a 90-cm^2 field (width and length) for 5 min while movements of the hindlimb were observed closely. Testing began 1 day after injury and then continued for 30 days. All rats were tested for functional deficits by trained investigators under double blind conditions.

Tissue Preparation

At chosen time points after SCI, rats were anesthetized with chloral hydrate and perfused via cardiac puncture with 0.1 M PBS, pH 7.4 and subsequently with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out and post-fixed by immersion in 4% paraformaldehyde overnight. The segment was embedded in paraffin or OCT compound as described previously (Du et al., 1999; Lee et al., 2000; Yune et al., 2003). Longitudinal sections were then cut at either 10 μm for paraffin-embedded tissues or at 8 μm for frozen tissues.

Lesion Area Analysis

After undergoing behavioral observation, rats at 18 and 28 days after SCI were used for lesion area analysis ($n = 3$ per group). To examine the lesioned area, serial 8-μm longitudinal sections through the central canal from vehicle or estradiol-treated rats were stained with Cresyl violet. The rostrocaudal boundaries of the tissue damage were defined by the presence of inflammatory cells, the loss of neurons, the existence of degenerating neurons, by microcyst formation and by gliosis as described previously (Beattie et al., 1997). The total lesion area (four serial sections per spinal cord) was determined by measuring the area of cavitation at the epicenter of the injury using a 2.5× objective and calculated by means of a Metamorphor imaging program (Universal Imaging Co., West Chester, PA).

TUNEL Staining

One day after SCI, rat spinal cords were prepared as described above. Serial 10 μm longitudinal sections through the anterior horn were selected and used for TUNEL-staining using an Apoptag in situ kit (Oncor, Gaithersburg, MD). Diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) was used as a substrate for peroxidase, and the sections were then counterstained with methyl green. Control sections were incubated in the absence of TdT enzyme. All TUNEL analyses were carried out by investigators who were blind as to the experimental conditions. Quantitation of TUNEL-positive cells was accomplished by counting the number of cells labeled positively using a 20× objective. Only those cells showing morphological features of nuclear condensation and/or compartmentation were counted as TUNEL-positive. All the cells stained positively within an area extending 2 mm rostral to 2 mm caudal to the lesion site were counted from each section (two sections from each spinal cord) without discriminating between gray and white matter or differentiating between neural and non-neural cells.

Immunostaining

Frozen sections were blocked in 5% normal serum and 0.1% Triton X-100 in TBS for 1 h at RT and then incubated with primary antibody (a monoclonal antibody against bcl-2; 1:100 dilution) overnight at 4°C, followed by HRP-conjugated secondary antibodies (Dako, Carpinteria, CA). The ABC method was used to detect labeled cells using a Vectastain kit (Vector Laboratories, Burlingame, CA). DAB served as the substrate for peroxidase. Some bcl-2 immunostains were double-labeled using antibodies to neuron specific enolase (NSE; 1:200 dilution; Chemicon, Temecula, CA). For double labeling, FITC (Amersham Biosciences, Arlington Heights, IL) or TRITC-conjugated secondary antibodies (Dako, Carpinteria, CA) were used. Also, nuclei were labeled with DAPI according to the protocol of the manufacturer (Molecular Probes, Eugene, OR). Images were collected using an Olympus microscope and SPOT™ (Diagnostic Instrument Inc). In all immunocytochemistry 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. Serial sections were also stained with Cresyl violet acetate for histological analysis.

Western Blot Analysis

At 24 h after injury, rats ($n = 3$ per group) were anesthetized and perfused via cardiac puncture with 0.1 M PBS, pH 7.4. Segments of spinal cord (5 mm) were isolated using the lesion site as the epicenter and the tissues were resuspended in 0.3 mL of lysis buffer [10 mM HEPES, 0.5 mM DTT, 10 mM KCl, and 1.5 mM $MgCl_2$ plus 10 μL each of protease inhibitors: 1 mg/mL pepstatin, 2.5 mg/mL leupeptin, 2 mg/mL aprotinin, and 0.2 M PMSF] and homogenized in a Dounce homogenizer. Tissue homogenate was centrifuged at 40,000 × g for 30 min at 4°C and the protein levels of the supernatant were determined using the BCA assay (Pierce, Rockford, IL). For analyses by Western blot, 50 μg of protein was separated by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride (Millipore, Bedford, MA) membranes by electrophoresis. The membranes were blocked with 5% nonfat skim milk in TBS for 1 h at room temperature and then incubated with a monoclonal antibody against bcl-2 overnight at 4°C. The membranes were then processed with HRP-conjugated anti-mouse secondary antibody (Pierce, Rockford, IL). Immunoreactive bands were visualized by chemiluminescence using Supersignal (Pierce, Rockford, IL). Experiments were repeated three times to ensure reproducibility.

DNA Laddering

At 24 h after injury, rats ($n = 3$ per group) were anesthetized and perfused via cardiac puncture with 0.1 M PBS, pH 7.4. Genomic DNA was isolated with a Qiagen genomic DNA kit (Qiagen, Germany) according to the manufacturer's instructions. In brief, the tissues were homogenized and incubated for 4 h in 10 mL of lysis buffer [7 M guanidine HCl, 30 mM Tris-HCl (pH 8.0), 30 mM EDTA, 5% Tween-20, 0.5% Triton X-100] with 5 mg of proteinase K (Sigma, St. Louis, MO) at 50°C. After incubation, genomic DNA was eluted with Qiagen genomic-tip 100/G and precipitated by adding 0.7 volumes of isopropanol at room temperature. The DNA was recovered by centrifugation at 7,000 × g for 30 min at 4°C and washed twice with 75% EtOH. The DNA pellet was dried and resuspended in 50 μL of 10 mM Tris-HCl (pH 8.5). Genomic DNA was labeled as described by Yakovlev et al. (1997). After quantification of DNA by spectrophotometer (Perkin-Elmer, Emeryville, CA), 1 μg of DNA was incubated for labeling in 20 μL of labeling mixture [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 10 mM $MgCl_2$] containing 2 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham Biosciences, Arlington Heights, IL) and 2.5 U of Taq DNA polymerase (Perkin-Elmer, Emeryville, CA) at room temperature for 20 min. The reaction was stopped by the addition of 2.5 μL of 0.5 M EDTA (pH 8.0). Radiolabeled DNA was loaded onto a 2% agarose gel, separated by electrophoresis at 50 V for 2.5 h in a 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA). After drying the gel, radiolabeled DNA in the dried gel was detected by exposure

to x-ray film. Experiments were repeated three times for reproducibility and relative intensity of DNA laddering was quantified using a ChemiImager™ 4400 (Alpha Innotech Co., San Leandro, CA).

Caspase-3 Activity

At 4 h after injury, rats ($n = 3$ per group) were anesthetized and perfused via cardiac puncture with 0.1 M PBS, pH 7.4. Caspase-3 enzyme activity was assayed using a procedure described by Springer et al. (1999). A segment of freshly dissected spinal cord 1 cm in length was Dounce-homogenized in 0.5 mL of homogenization buffer (pH 7.4) containing 10 mM HEPES, 250 mM sucrose, 1 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 10 μL each of 1 mg/mL pepstatin, 2.5 mg/mL leupeptin, 2 mg/mL aprotinin, and 0.2 M PMSF. The samples were centrifuged and the protein levels of the supernatant were determined using the BCA assay (Pierce, Rockford, IL). One hundred micrograms of cytosolic fraction of spinal cord protein was added to 1 mL of caspase homogenization buffer containing 15.0 μM Z-DEVD-AFC (Enzyme Systems Products, Livermore, CA). Samples were incubated at room temperature for 5 min, and relative fluorescence (excitation at 400 nm and emission at 505 nm) was measured for 1 h using a K2 multi-frequency phase fluorometer (ISS Inc., Champaign, IL). The specific activity of the samples was calculated relative to a standard curve using recombinant caspase-3 (Upstate Biotechnology, Lake Placid, NY).

RNA Isolation and RT-PCR

At 1, 6, 12, and 24 h after injury, rats ($n = 3$ per group) were anesthetized and perfused via cardiac puncture with 0.1 M PBS, pH 7.4. Total RNA was isolated using Trizol Reagent (GibcoBRL, Gaithersburg, MD) according to the manufacturer's instructions. To ascertain that all RNA samples would be DNA-free, samples were treated with RNase-free DNase I (Sigma, St. Louis, MO). After spectrophotometric quantification, the purified RNA was separated on a formaldehyde-agarose gel to check the extent of degradation. One microgram of total RNA was reverse-transcribed into first strand cDNA in each 20 μL of reaction mixture, using MMLV reverse transcriptase (GibcoBRL, Gaithersburg, MD) according to the manufacturer's instructions. A 20-μL PCR reaction contained 2 μL of first-strand cDNA, 0.6 U of Amplitaq polymerase (Perkin-Elmer, Branchburg, NJ), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM dNTP, and 10 pmol of each specific primer. Samples were subjected to 25–35 cycles of 95°C for 1 min, 52–60°C for 1 min, and 72°C for 2 min on a thermocycler (Perkin-Elmer, Emeryville, CA). The primers used for this experiment were designed according to the sequences previously reported for *bcl-2*, *bax*, *bcl-x* (*L & S*) (Greenlund et al., 1995) and *bad*. The β-actin primer, which was used as an internal control, was designed according to the sequence previously reported (Nudel et al., 1983). The primer sequences were as follows: *bcl-2* sense primer, 5′-CTGTACGGCCCCAGCATGGCG-3′ and antisense primer, 5′-GCTTTGTTTCATGGTACATC-3′ (231 bp); *bax* sense primer, 5′-GGGAATTCTGGAGCTGCAGAGGATGATT-3′ and antisense primer, 5′-GCGGATCCAAGTTGCCATCAGCAAACAT-3′ (96 bp); *bcl-x* (*L & S*) sense primer, 5′-AGGCTGGCGATGAGTTTGAA-3′ and antisense primer, 5′-CGGCTCTCGGCTGCTGCATT-3′ (*bcl-x_L* 337 bp; *bcl-x_S* 150 bp); *bad* sense primer, 5′-CACTCCCTAGGCTTGAGGAA-3′ and antisense primer, 5′-TCCTGCTCACTCGGCTCAAA-3′ (209 bp); β-actin sense primer, 5′-ATTTGGCACCACACTTTCTACA-3′, and antisense primer, 5′-TCACGCACGATTTCCCTCTCAG-3′ (245 bp). Negative controls included PCR reactions lacking primers or reverse transcriptase. After amplification, RT-PCR products were separated on a 1.5–2% agarose gel containing 0.5 μg/mL ethidium bromide. The amplified cDNA fragments were visualized under ultraviolet light. Densitometry readings of gel bands were performed using a ChemiImager™4400 (Alpha Innotech Co, San Leandro, CA). 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 ± SD values. Quantitative data from open field locomotor scores and lesion areas were evaluated for statistical significance by two-way ANOVA with replications following Duncan's test for multiple comparisons; data from TUNEL-positive cells, caspase-3 activities, and RT-PCR and Western analyses were also evaluated for statistical significance using two-way ANOVA with a *post hoc* Tukey test. In all analyses, a p value of <0.05 was considered statistically significant.

RESULTS

17β-Estradiol Improves Functional Recovery after SCI

The effect of estradiol on locomotor function was determined to see whether the drug had beneficial effects on functional recovery after SCI. Estradiol (3, 100, and 300 μg/kg) was administrated intravenously 1–2 h prior to SCI (*pre-treatment*). Immediately after SCI, all injured rats had paralyzed hind limbs. Functional recovery was

assessed in rats by the BBB test, a standard method for assessing hind limb motor function after SCI (Basso et al., 1996 a,b). As shown in Figure 1, the BBB scores were significantly higher in rats treated with 100 μg/kg of estradiol at 15–30 days after SCI than in vehicle-treated rats. For example, 30 days after injury, BBB scores of estradiol-treated (100 μg/kg) and vehicle-treated rats were 18.3 $\pm$ 0.6 ($p < 0.001$) and 15 $\pm$ 1, respectively.

17β-Estradiol Reduces the Lesion Area After SCI

We examined the effect of 17β-estradiol treatment on the size of the lesion area after SCI to determine whether it ameliorates tissue necrosis after injury. Within hours of an acute SCI, histological examinations of the injured tissues revealed that the loss of neuronal and glial cells was evident within the epicenter of the lesion site with a breakdown of axonal segments in white matter. Thereafter, the lesion size was found to be increased progressively in spinal cords treated either with vehicle or with 17β-estradiol (Lee et al., 2004). However, the lesion area in spinal cords treated with 17β-estradiol (100 μg/kg) was significantly reduced at both 18 and 28 days after SCI when compared to vehicle controls (Fig. 2). At 28 days after injury, for example, the lesion area in injured spinal cords treated with estradiol and with vehicle measured 2.06 $\pm$ 0.2 mm^2 ($p < 0.05$) and 2.5 $\pm$ 0.3 mm^2, respectively.

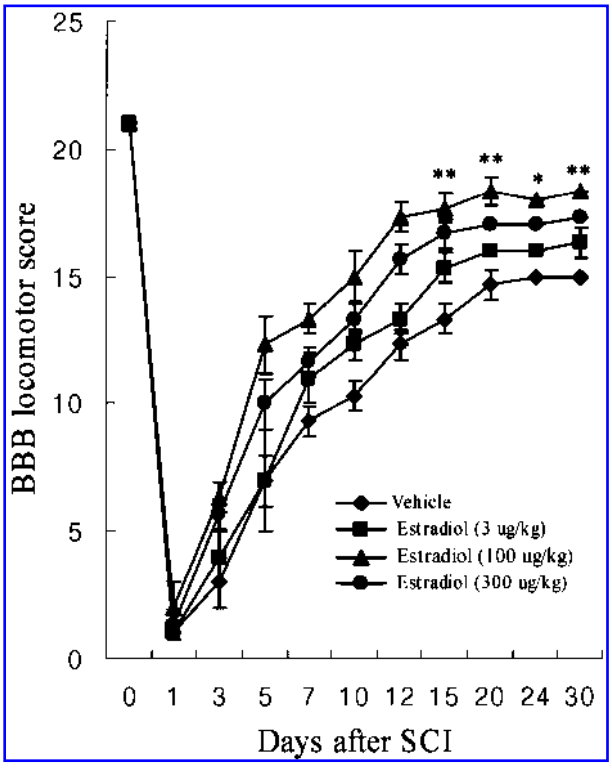

FIG. 1. Effect of 17β-estradiol (*pre*-treatment) on functional recovery following SCI. Functional recovery was assessed by BBB behavioral testing which began 1 day after injury and then continued for 30 days as described in the Materials and Methods section. Note that the BBB scores after SCI were significantly higher in rats treated with estradiol (100 μg/kg) at 15 to 30 days as compared to vehicle-treated rats ($n = 8$ per group). Data represent mean $\pm$ SD. $^{*}p < 0.05$, $^{**}p < 0.001$ vs. vehicle.

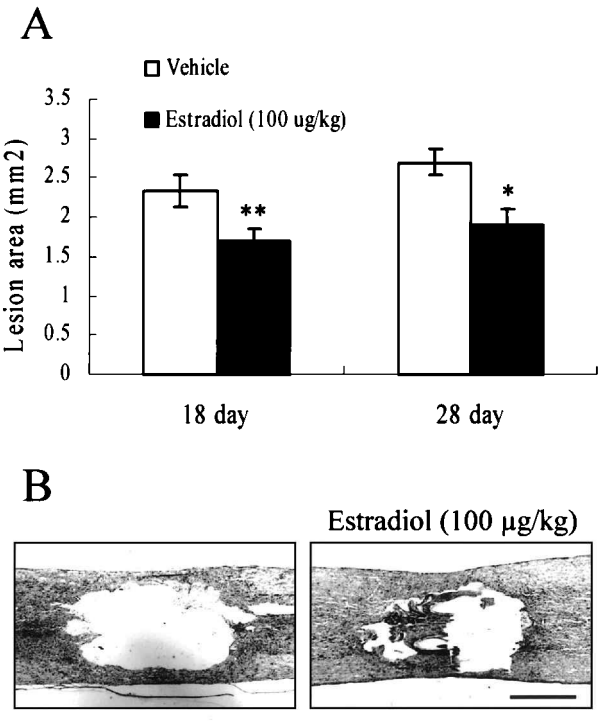

FIG. 2. Effect of estradiol on the lesion area after spinal cord injury. 17β-estradiol (100 μg/kg) or vehicle were administered intravenously to rats 1 hr prior to injury and the animals were sacrificed 18 and 28 days after SCI. Total lesion areas were determined by calculating the area of cavitation at the epicenter of the injury as described in the Materials and Methods. **(A)** Lesion areas at 18 and 28 days after SCI. Data represent mean $\pm$ SD ($n = 3$ for each time point). Note that treatment with estradiol significantly reduced the lesion size after SCI as compared to that observed in the vehicle control. $^{*}p < 0.05$, $^{**}p < 0.001$ versus vehicle. **(B)** Representative photographs of spinal cord lesion areas in longitudinal sections taken 28 days after SCI for both vehicle- and estradiol-treated spinal cords. Bar = 1 mm.

17β-Estradiol Treatment Reduces Apoptotic Cell Death

Acute spinal cord injury induces extensive apoptotic cell death of neurons and glial cells (Beattie et al., 1997; Crowe et al., 1997; Emery et al., 1998; Lee et al., 2000; Li et al., 1996; Liu et al., 1997; Shuman et al., 1997; Yong et al., 1998; Yune et al., 2003). Since estrogen ex-

erts neuroprotective effects on CNS neurons exposed to a variety of insults (Garcia-Sergura et al., 2001), we examined whether the drug reduced apoptotic cell death after SCI. 17β-estradiol was administered intravenously 1–2 h prior to SCI, and apoptotic cell death was analyzed by TUNEL staining and DNA gel electrophoresis. As shown in Figure 3A,B, estradiol treatment (100 and 300 μg/kg) significantly reduced the number of TUNEL-positive cells 24 h after SCI as compared to those cells in the vehicle control. For example, the number of TUNEL-positive cells in 17β-estradiol (100 μg/kg) and vehicle-treated spinal cords was 50 $\pm$ 5 and 134 $\pm$ 8 ($p < 0.001$), respectively. DNA gel electrophoresis also revealed a marked decrease in DNA laddering following estradiol treatment (Fig. 3C).

17β-Estradiol Reduces Caspase-3 Activity

Recent evidence shows that activation of caspase-3 is involved in apoptotic cell death as a common executive molecule after SCI (Emery et al., 1998; Matsushita et al., 2000; Springer et al., 1999). Since 17β-estradiol treatment reduced apoptosis after SCI as revealed by TUNEL staining and DNA gel electrophoresis (Fig. 3), we examined whether estradiol also alleviates caspase-3 activity after SCI. As shown in Figure 4, estradiol treatment (100 μg/kg) significantly reduced specific caspase-3 activity 4 h after SCI as compared to that of the vehicle control (5.7 $\pm$ 0.81 vs. 9.8 $\pm$ 0.95, $p < 0.001$).

17β-Estradiol Increases Expression of bcl-2 and bcl-x after SCI

Recent evidence indicates that estrogen protects neurons via a variety of mechanisms (Behl et al., 1997; Culmsee et al., 1999; Dubal et al., 1999; Garcia-Sergura et al., 2001; Gridley et al., 1998; Honda et al., 2000; Sawada et al., 1998; Singer et al., 1999). Recent reports show that its neuroprotective effects may be attributable

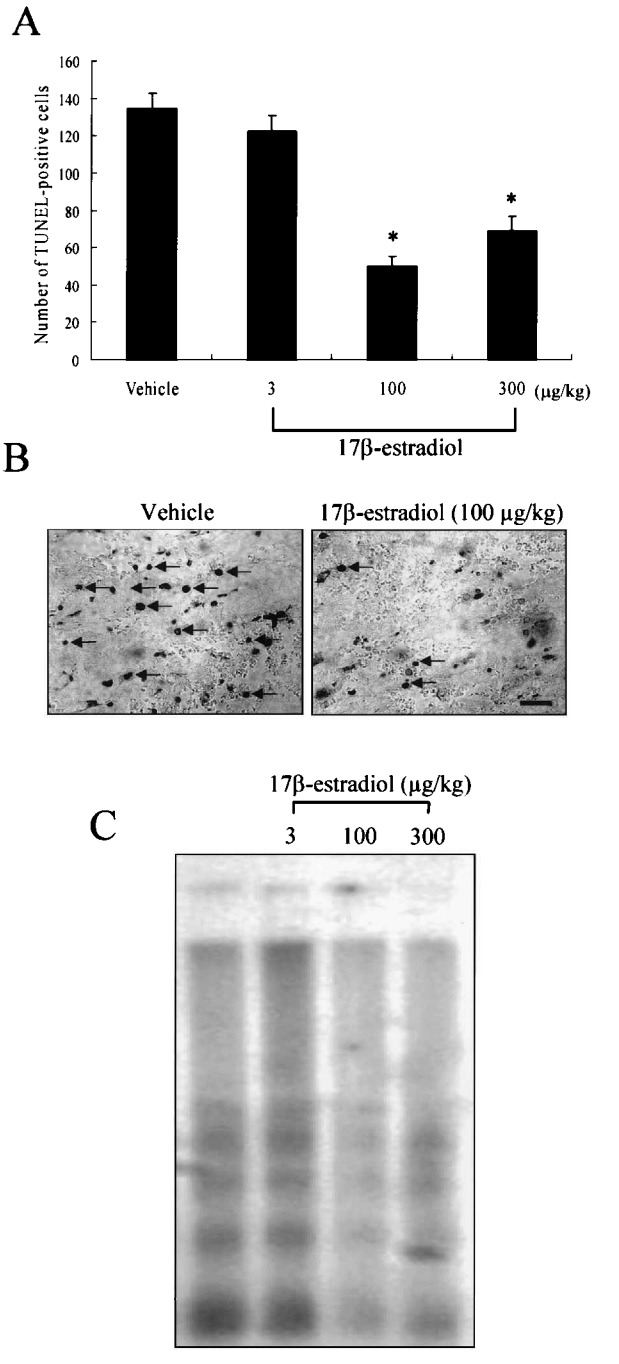

FIG. 3. Effect of 17β-estradiol on apoptotic cell death after SCI. Rats were treated with vehicle or estradiol (100 μg/kg) 1 h prior to injury, and spinal cord tissues were then collected 24 h post-injury. Longitudinal sections were processed for TUNEL staining, and TUNEL-positive cells were counted as described in the Materials and Methods. **(A)** TUNEL-positive cells in injured spinal cords. Data represent mean $\pm$ SD (n = 3). Note that 17β-estradiol treatment after SCI significantly reduced the number of TUNEL-positive cells as compared to the positive cells in the vehicle-treated control. $^{*}p < 0.001$. **(B)** Representative photographs of TUNEL-positive cells (arrows) 24 h after SCI treated with vehicle or estradiol (100 μg/kg). Bar = 30 μm. **(C)** DNA gel electrophoresis 24 h after SCI. DNA was isolated from the spinal cord tissues, labeled at the 3′ end with [^{32}P]deoxycytidine triphosphate (dCTP) and separated by gel electrophoresis as described in the Materials and Methods. Lane 1, treated with vehicle; Lane 2, 3 and 4, treated with estradiol (3, 100, and 300 μg/kg). Note that DNA laddering was markedly decreased following treatment with 100 μg/kg of estradiol after SCI when compared to the laddering seen in the vehicle control. The gel presented is a representative of results from three separate experiments.

in part to a modulation of expression of anti-apoptotic genes such as bcl-2 and bax (Alkayed et al., 2001; Dubal et al., 1999; Goodman et al., 1998). Therefore, we examined the effect of 17β-estradiol on expression of bcl-2 family members after SCI. As shown in Figure 5, estradiol treatment (3, 100, and 300 μg/kg) significantly increased the level of Bcl-2 protein at 24 h after SCI. RT-PCR analysis also revealed that estradiol treatment (100 μg/kg) significantly increased expression of bcl-2, bcl-x_L and bcl-x_S at 6 and 12 h after SCI as compared to those of vehicle controls (Fig. 6). However, estradiol treatment had no significant effect on the mRNA levels of bad and bax, known pro-apoptotic genes, as compared to those of vehicle (Fig. 6). Also, immunohistochemical staining revealed that estrogen treatment increased expression of Bcl-2 protein after SCI. As shown in Figure 7A, little or no Bcl-2 immunoreactivity was observed in the sham-operated spinal cord. In the vehicle-treated spinal cord 24 h after injury, however, weak Bcl-2 immunoreactivity was detected in presumptive neurons within the gray matter in the lesion area (data not shown). As expected, intense immunoreactivity to Bcl-2 was seen in presumptive neurons in the gray matter of the estradiol-treated spinal cord—even in those cells located several mm both rostrally and caudally from the lesion (Fig. 7B). Double staining revealed that Bcl-2-positive cells in the gray matter were co-localized with NSE, a neuronal marker (Fig. 7C–F)

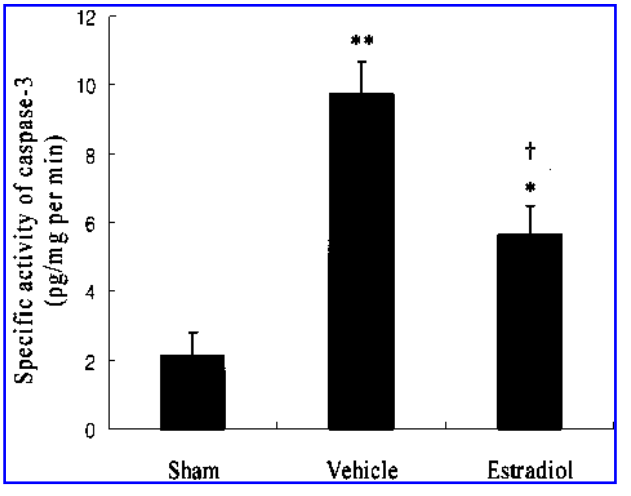

FIG. 4. Effect of 17β-estradiol on caspase-3 activity after SCI. Estradiol (100 μg/kg) was administered intravenously to rats 1 h prior to injury and the animals were sacrificed 4 h after injury. Fluorogenic caspase activity was measured as described in Materials and Methods. Note that caspase-3 specific activity was significantly reduced in animals receiving estradiol treatment (5.7 ± 0.81) as compared to vehicle controls (9.8 ± 0.95). Values represent mean ± SD ($n = 3$ per group). $^{*}p < 0.01$, $^{**}p < 0.005$ versus sham; $^{\dagger}p < 0.001$ versus vehicle.

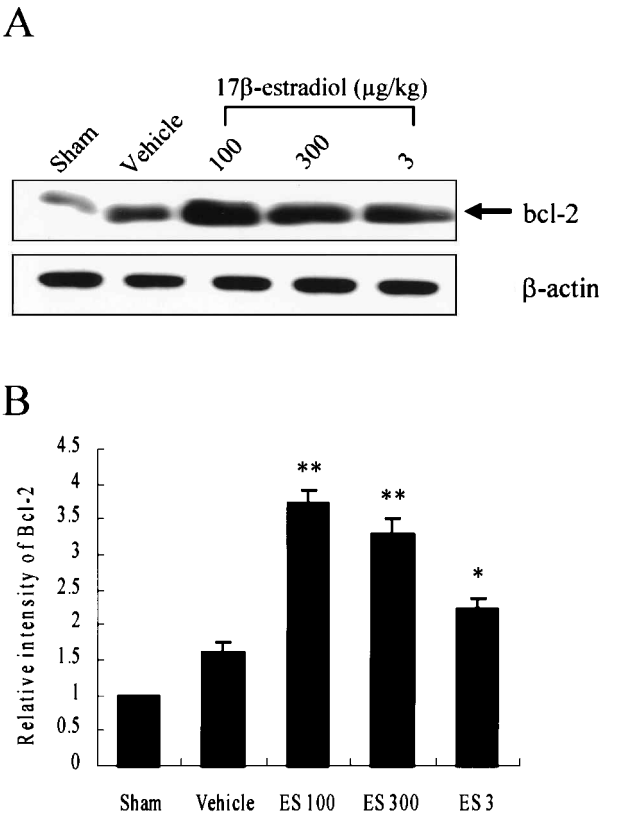

FIG. 5. Effect of 17β-estradiol on expression of Bcl-2 after SCI. Vehicle and estradiol (3, 100, and 300 μg/kg) were administered intravenously 1 h prior to injury, and spinal cord tissues were collected 24 h post-injury ($n = 3$ per group). Western blot analysis was performed as described in the Materials and Methods. Note that the increased Bcl-2 protein found after treatment with 100 μg/kg of estradiol was greater than that found after treatment with 3 or 300 μg/kg of the drug. **(A)** Western blot analysis of Bcl-2 expression in sham-operated, vehicle- and estradiol-treated spinal cords. The gels presented are representative of results from three separate experiments. **(B)** Densitometry readings of gel bands expressed as arbitrary units of relative intensities to that of sham control. Values represent mean ± SD of three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.001$ versus vehicle.

17β-Estradiol Administration after Injury Also Improves Functional Recovery after SCI

Finally, in order to assess whether this steroid holds any potential in a more therapeutic mode, we decided to test it by administering it immediately after injury. As shown in Figure 8, the BBB scores were significantly higher in estradiol-treated (100 μg/kg) rats 19 to 30 days

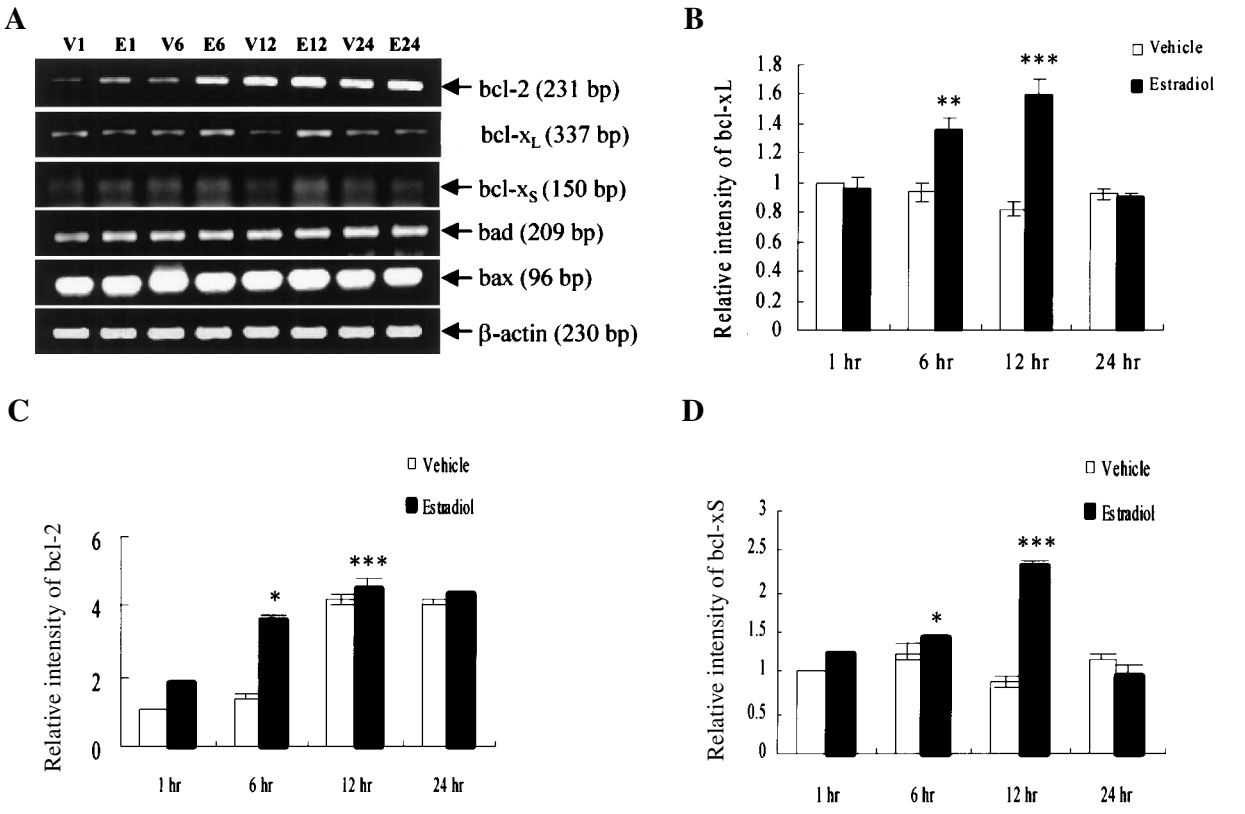

FIG. 6. Effects of 17β-estradiol on expression of Bcl-2 family members after SCI. 17β-estradiol (E; 100 μg/kg) or vehicle (V) was administered intravenously to rats 1 hr prior to injury, and spinal cord tissues were collected 1, 6, 12, and 24 h after injury (n = 3 per group). Total RNA isolation and RT-PCR was performed as described in the Materials and Methods section. **(A)** RT-PCR analyses of bcl-2, bcl-x_L, bcl-x_S, bad, and bax mRNA expression after SCI. Note that estradiol treatment significantly increased the bcl-2 and bcl-x mRNA expression after SCI as compared to that of vehicle control, while the bax or bad expression was not affected by the drug treatment. The gels presented are representative of results from three separate experiments. **(B–D)** Densitometry readings of gel bands were expressed as arbitrary units as relative intensities to that of vehicle control. Values are mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle.

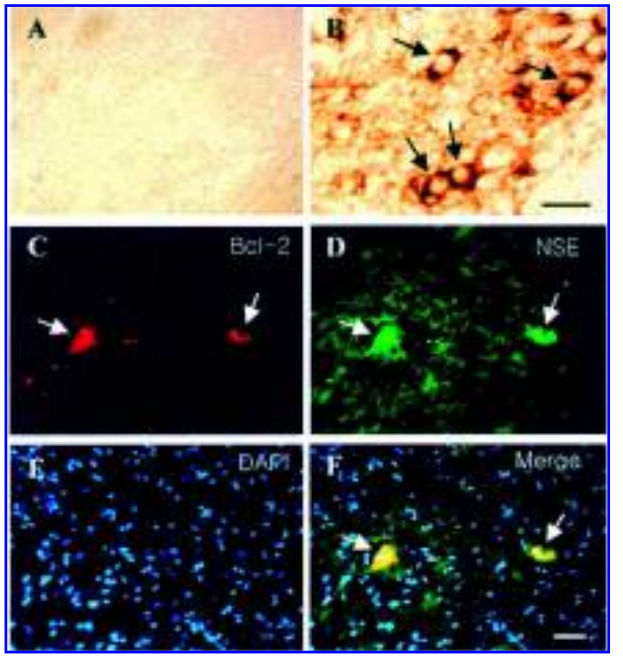

FIG. 7. Immunocytochemical detection of Bcl-2 protein after spinal cord injury. 17β-estradiol (100 μg/kg) and vehicle were administered intravenously to rats (n = 2 per group) 1 h prior to injury and spinal cord tissues were collected 24 h after injury. **(A)** Bcl-2 immunoreactivity in the sham-operated spinal cord. Note that little or no Bcl-2 immunoreactivity was detected in the spinal cord. **(B)** Bcl-2 immunoreactivity in the gray matter of the estradiol-treated spinal cord 24 h after injury. Note that intense Bcl-2 immunoreactivity was seen in presumptive neurons in the gray matter. **(C–F)** Double immunostaining for Bcl-2 with NSE. Bcl-2-positive cells (**C,** see arrows) in the gray matter were co-localized with NSE (**D,** see arrows) and labeled its nucleus with DAPI (**E**) 24 hr after SCI (**F,** merged image, see arrows). Bar = 30 μm.

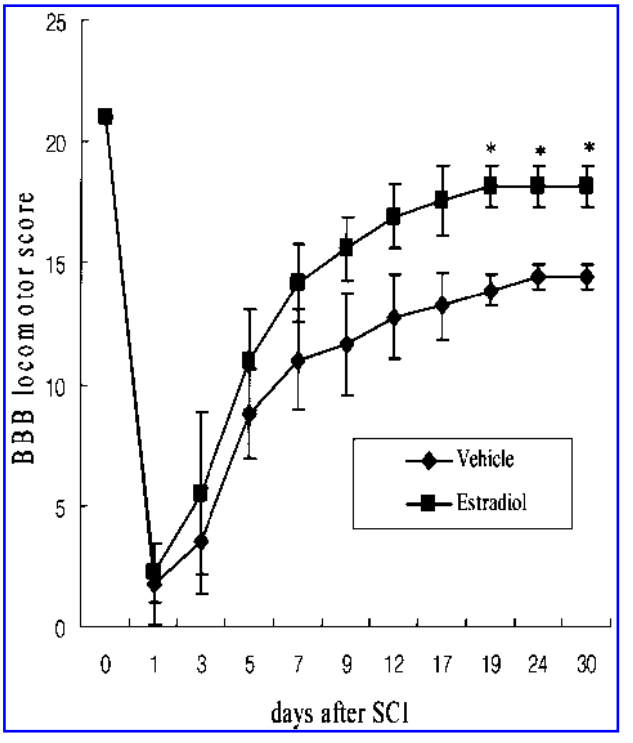

FIG. 8. Effect of 17β-estradiol (post-treatment) on functional recovery following SCI. Rats received intravenous injections of estradiol immediately after injury, and functional recovery was assessed by BBB behavioral testing as described in the Materials and Methods section. Note that the BBB scores after SCI were significantly higher in rats treated with estradiol (100 μg/kg) at 19 to 30 days as compared to vehicle-treated rats (n = 8 per group). Data represent mean $\pm$ SD. $^{*}p < 0.001$ versus vehicle.

after SCI as compared to those in vehicle-treated rats. At 30 days after injury, for example, BBB scores of estradiol-treated and vehicle-treated rats were 18.2 $\pm$ 0.8 ($p <$ 0.001) and 14.4 $\pm$ 0.5, respectively.

DISCUSSION

The primary objective of this study was to assess the long-term effect of 17β-estradiol on locomotor function after SCI. Here, we report for the first time the beneficial effects of estradiol on functional recovery after SCI. Three essential findings of these experiments are that (i) 17β-estradiol treatment attenuates lesion size and improves functional recovery after SCI; (ii) treatment with the drug reduces apoptotic cell death and caspase-3 activity after SCI; and (iii) estradiol treatment increases expression of bcl-2 and bcl-x after SCI. These results indicate that the beneficial effects of estradiol treatment may be linked to its neuroprotective activity against injury-induced apoptosis, thereby reducing the size of lesions and improving functional recovery after SCI. Furthermore, our data show that the neuroprotective effect of 17β-estradiol might be mediated in part by the modulation of bcl-2 and bcl-xl expression after SCI.

Caspases are known to be involved in neuronal apoptosis in CNS injury and neurodegenerative diseases (Friedlander and Yuan, 1998; Li and Wong, 2000; Ona et al., 1999; Schielke et al., 1998; Yakovlev and Faden, 2001; Yuan and Yankner, 2000). Also, caspase-3 has been implicated in apoptotic cell death after SCI (Citron et al., 2000; Li et al., 2000; Nesic et al., 2001; Springer et al., 1999, 2000), although upstream activators were not yet fully elucidated. The present study showed that 17β-estradiol treatment reduced not only apoptotic cell death but also caspase-3 activity after SCI. Thus, our results suggest that the neuroprotective effect of estradiol may be mediated, in part, by inhibition of caspase-3–dependent apoptosis. Although estradiol is known to inhibit the caspase cascade upstream (MacManus and Linnik, 1997), the precise action of the drug on the caspase cascade—whether it be on the cytochrome c-mediated mitochondrial (caspase-9) or the death receptor-mediated extramitochondrial (caspase-8) apoptotic pathway—has yet to be determined. Nevertheless, the present study supports the view that estrogen treatment attenuates apoptotic cell death by reducing caspase-3 activity induced by a variety of insults (Dare et al., 2000; Jover et al., 2002; Sawada et al., 2000; Thompson et al., 2002).

Estrogen exerts its protective effects on CNS neurons via two basic mechanisms: (i) via effects mediated by the activation of estrogen receptors (ERs) (Dubal et al., 1999; Honda et al., 2000; Singer et al., 1999) or (ii) effects independent of the activation of ERs (Behl et al., 1997; Culmsee et al., 1999; Gridley et al., 1998; Sawada et al., 1998). Some of the ER-independent mechanisms underlying the neuroprotective effects of estrogen, for example, relate to its anti-oxidant activities and the modulation of neurotransmitter receptor function (Culmsee et al., 1999; Sawada et al., 1998). For ER-mediated mechanisms, estrogen activity is mediated by its cognate receptor (ER); occupancy of the ER by the hormone induces conformational changes which allow its interaction with specific enhancers known as estrogen-responsive elements (EREs) and with general transcription factors (Beato, 1989). Several ER-mediated mechanisms have been reported such as the modulation of the Bcl-2 family (Alkayed et al., 2001; Dubal et al., 1999), activation of phosphatidylinositol 3-kinase (Honda et al., 2000), mitogen-activated protein kinase (Singer et al., 1999), or induction of heat shock proteins (Lu et al., 2002). Singer et al. (1998) showed that an increased expression of Bcl-

2 is associated with the neuroprotective activity of estrogen against H_2O_2, or glutamate-induced toxicity in NT2 neurons. Pike et al. (1999) also showed that estrogen significantly increases expression of bcl-x_L, an anti-apoptotic gene, in cultured hippocampal neurons; furthermore, its expression is associated with a reduction in apoptosis induced by beta-amyloid. The present experiments showed that 17β-estradiol treatment significantly increased expression of bcl-2 and bcl-x but had no significant effect on bad and bax expression. Our results, taken together with reports by others (Alkayed et al., 2001; Dubal et al., 1999; Singer et al., 1998), suggest that the neuroprotective effects of 17β-estradiol might be mediated in part by regulating the expression of anti-apoptotic genes.

Physiological levels of estradiol act through genomic mechanisms to protect neurons against ischemic insults since the actions of the drug are not rapid and require pretreatment (Dubal et al., 1998, 1999), while high concentrations of the hormone may protect neurons from oxidative stress via non-genomic mechanisms (Garcia-Segura et al., 2001; Behl et al., 1997). It is known that high "super" physiologic concentrations of estradiol, like the doses used in the present experiments, act through non-genomic mechanisms as by anti-oxidant activity to protect neurons against insults (Garcia-Segura et al., 2001; Behl et al., 1997). Chen et al. (1998) reported that high doses of 17β-estradiol improve the histological outcome after transient forebrain ischemia in hippocampal CA1 pyramidal cells of gerbils while low doses of the drug show no neuroprotective effect. By contrast, Sudo et al. (1997) showed that estrogen decreased ischemic damage in gerbils at a low dose (0.25 μg/day), whereas a high dose (1.25 μg/day) was ineffective against ischemic damage. Furthermore, Dubal et al. (1998) reported that estrogen is neuroprotective at both low (10 pg/mL in plasma) and high (100 pg/mL in plasma) levels in the rat cerebral cortex after ischemia. The present experiments showed that the highest neuroprotective effects were observed with treatment with 100 μg/kg of estradiol while a low (3 μg/kg) or a high (300 μg/kg) dose showed no significant neuroprotective effect. These results indicate some discrepancies concerning the optimal dose of estrogen exerting its neuroprotective effects *in vivo*. However, further studies are necessary to determine the precise mechanism(s) by which estrogen provides neuroprotection against SCI. Finally, our results showed that when 17β-estradiol (100 μg/kg) was administered immediately after SCI (*post-treatment*), the drug also significantly improved the hind limb motor function 19–30 days after SCI as compared to that vehicle-treated control. Thus, it is clear that systemic administration of exogenous estradiol either prior to injury (*pre-treatment*) or after injury (*post-treatment*) provides neuroprotection against CNS injury, possibly through either genomic or/and nongenomic mechanisms. This study raises the possibility that estradiol could be a useful therapy in preventing cell death thereby improving functional recovery after SCI, even if administered after injury.

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