17β-Estradiol Inhibits MMP-9 and SUR1/TrpM4 Expression and Activation and Thereby Attenuates BSCB Disruption/Hemorrhage After Spinal Cord Injury in Male Rats

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Blood-spinal cord barrier (BSCB) disruption and progressive hemorrhage after spinal cord injury (SCI) lead to secondary injury and the subsequent apoptosis and/or necrosis of neuron and glia, causing permanent neurological deficits. In this study, we examined the effect of 17β-estradiol (E2) on BSCB breakdown and hemorrhage as well as subsequent inflammation after SCI. After a moderate contusion injury at the 9th thoracic segment of spinal cord, E2 (300 μg/kg) was administered by iv injection immediately after SCI, and the same dose of E2 was then administered 6 and 24 hours after injury. Our data show that E2 attenuated BSCB permeability and hemorrhage and reduced the infiltration of neutrophils and macrophages after SCI. Consistent with this finding, the expression of inflammatory mediators was significantly reduced by E2. Furthermore, E2 treatment significantly inhibited the expression of sulfonylurea receptor 1 and transient receptor potential melastatin 4 after injury, which are known to mediate hemorrhage at an early stage after SCI. Moreover, the expression and activation of matrix metalloprotease-9 after injury, which is known to disrupt BSCB, and the degradation of tight junction proteins, such as zona occludens-1 and occludin, were significantly inhibited by E2 treatment. Furthermore, the protective effects of E2 on BSCB disruption and functional improvement were abolished by an estrogen receptor antagonist, ICI 182780 (3 mg/kg). Thus, our study provides evidence that the neuroprotective effect of E2 after SCI is, in part, mediated by inhibiting BSCB disruption and hemorrhage through the down-regulation of sulfonylurea receptor 1/transient receptor potential melastatin 4 and matrix metalloprotease-9, which is dependent on estrogen receptor. (Endocrinology 156: 1838–1850, 2015)

Traumatic spinal cord injury (SCI) is a devastating condition that results in a permanent disability. The blood-spinal cord barrier (BSCB) is the functional equivalent of the blood-brain barrier (BBB) that provides a specialized microenvironment for the cellular constituents of the spinal cord. When the BSCB is damaged by an injury, blood cells cross into the injured parenchyma and contribute to secondary injuries such as inflammation (1–3). These secondary injuries induce apoptosis of neurons and glia, which results in permanent neurological deficits (4–6). It has been well established that the elevated expression and activation of matrix metalloproteases (MMPs) can be detrimental and can result in numerous pathological conditions, including BBB or BSCB disruption after ischemic brain injury and SCI (4, 5, 7, 8). Additionally, progressive hemorrhage followed by necrotic cell death occurs at an early stage after SCI, and this
event is known to be mediated by sulfonylurea receptor (SUR)-regulated Ca\(^{2+}\)-activated, [ATP](i)-sensitive non-specific cation channels (9). The report by Simard et al (10) also showed that the suppression of \textit{Abcc8}, which encodes SUR1, attenuates the hemorrhagic necrosis of capillary endothelial cells after autodestruction after SCI.

Estrogen is known as a potential therapeutic agent for the treatment of many neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, stroke, and brain trauma, because it has broad neuroprotective effects against a variety of insults to the central nervous system (11–14). Our previous studies showed that 17\(^{-}\)-estradiol (E2) improves functional recovery by inhibiting apoptosis of neurons after SCI (15) and that this effect is mediated by phosphoinositide-3-kinase/Akt-induced cAMP response element-binding protein-dependent Bcl-2 expression (16). Additionally, we have also shown that estrogen inhibits oligodendrocyte cell death by attenuating Ras homolog gene family, member A-c-Jun N-terminal kinases 3 activation after SCI (17). Furthermore, some studies have shown that the neuroprotective effect of estrogen is mediated by inhibiting BBB disruption and hemorrhagic transformation in experimental ischemic and traumatic brain injury models (18–20). The report by Tomás-Camardiel et al (21) showed that estrogen treatment in ovariectomized animals protects against lipopolysaccharide-induced BBB disruption.

Here, we examined whether E2 prevents BSCB disruption and/or hemorrhage and thereby inhibits inflammation after SCI. We found that E2 significantly inhibited BSCB disruption and hemorrhage, as well as the subsequent blood cell infiltration and inflammation, by inhibiting the up-regulation of SUR1/transient receptor potential melastatin 4 (TrpM4) and MMP-9 expression and activation after SCI, which is mediated through an estrogen receptor (ER).

**Materials and Methods**

**Spinal cord injury**

Adult rats (Sprague-Dawley; male; 250–300 g; Sam:TacN (SD) BR, Samtako) 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 spinous processes of T8 and T11 were then clamped to stabilize the spine, and the exposed dorsal surface of the cord was subjected to moderate contusion injury (25 g/cm) using a New York University impactor as previously described (22). For the sham-operated controls, rats underwent a T10 laminectomy without weight-drop injury. Throughout the surgical procedure, body temperature was maintained at 37 ± 0.5°C with a heating pad. Postoperatively, rats were received sc supplemental fluids (5 mL, lactated ringer) and antibiotics (gentamicin, 5 mg/kg, im injection) once daily for 5 days after surgery. The bladder was emptied manually 3 times per day until reflexive bladder emptying was established. 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 treatment**

E2 (2-hydroxypropyl-\(\beta\)-cyclodextrin-encapsulated; Sigma-Aldrich) was dissolved in sterile 0.1M PBS (pH 7.4), and rats received iv injection of E2 (300 \(\mu\)g/kg) or \(\beta\)-cyclodextrin solution immediately after SCI and then at 6 and 24 hours with the same dose. Because our previous report showed that 300 \(\mu\)g/kg is an optimal dose for functional recovery after SCI (17), we used this concentration of drug throughout experiments. The vehicle (Veh) control group received injections of \(\beta\)-cyclodextrin in PBS with same interval. An antagonist of ER, ICI 182780 (Tocris) was dissolved in 50% dimethylformamide in 0.9% saline and was given ip injection (3 mg/kg, ip) before each injection of E2. The Veh control group received injections of 50% dimethylformamide in 0.9% saline.

**Evans blue assay**

At 24 hours after SCI, the permeability of the BSCB was investigated with Evans blue dye extravasation as previously described (17). In brief, 5 mL of 2% Evans blue dye (Sigma) was administered via ip injection at 1 day after SCI. Three hours later, rats were anesthetized and perfused with saline by intracardiac perfusion. To quantify Evans blue extravasation, the T9 spinal cord segment (3 mm), including lesion epicenter was removed and homogenized in a 50% trichloroacetic acid solution. The Evans blue level was determined by spectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. Dye in samples was determined as micrograms per gram of tissue from a standard curve plotted using known amounts of dye. To determine the effect of delayed administration of E2 on BSCB disruption, E2 (300 \(\mu\)g/kg) was administered via iv at 1, 2, or 6 hours after SCI and then injected at 6 hours after first injection. The Veh control group received equal volume iv injections of \(\beta\)-cyclodextrin in PBS at the corresponding time points.

**Spectrophotometric assay for intraspinal hemorrhage**

The hemoglobin content in the injured spinal cord tissue was quantified with a spectrophotometric assay as described (10). Rats were perfused with heparinized saline to remove intravascular blood, and 5 mm segments of cord encompassing the lesion site were homogenized and sonicated on ice with a pulse ultrasonicator (Ultrasonics Co) for 1 minute. After centrifugation at 16 000g for 30 minutes, the hemoglobin-containing supernatant was collected and allowed to stand for 15 minutes. This reaction converts hemoglobin to cyanomethemoglobin, which has an absorbance at 540 nm, and whose concentration can then be assessed by the optical density of the solution at 550 nm. To validate whether the measured absorbance following these procedures reflects the amount of hemoglobin, known quantities of bovine erythrocyte hemoglobin (Sigma) were analyzed with same procedures alongside every spinal cord tissue assay. As an additional measure, blood was obtained from control mice by cardiac puncture after anesthesia. Incremental aliquots of this
blood were then added to freshly homogenized spinal cord tissue obtained from injured rats with or without E2 treatment to generate a standard absorbance curve.

**Tissue preparation**

At specific time points after SCI, rats were anesthetized with chloral hydrate and perfused via cardiac puncture initially with 0.1M PBS and subsequently with 4% paraformaldehyde in 0.1M PBS. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out, postfixed by immersion in the same fixative (4% paraformaldehyde) for 5 hours, and placed in 30% sucrose in 0.1M PBS. The segment was embedded in Optimal Cutting Temperature compound for frozen sections, and longitudinal or transverse sections were then cut at 10 μm on a cryostat (CM1850; Leica). For molecular work, rats were perfused previously described (23). For quantification of MPO or ED-1 immunohistochemistry with antibodies against myeloperoxidase (MPO) (1:100; Dako) and ED-1 (1:1000; Serotec) as previously described (23). For quantification of MPO or ED-1 intensity, serial transverse sections (20-μm thickness) were collected every 100-μm section rostral and caudal 3000 μm to the lesion site (total 60 sections). Digital images of MPO- or ED-1-stained tissues were obtained, and we quantified the entire fluorescent intensity of the each transverse section above threshold by using MetaMorph software (Molecular Devices) and averaged. The threshold value was at least 3 times the background, and the background was quantified and normalized to the primary antibody omitted control. Immunostaining control studies were performed by omission of the primary antibodies, by replacement primary antibodies with nonimmune, control antibody, and by preabsorption with an excess (10 μg/mL) of the respective antigens. Serial sections were also stained for histological analysis with Cresyl violet acetate.

**Immunohistochemistry**

The spinal cord segment was embedded in Optimal Cutting Temperature compound for frozen sections, and longitudinal or transverse sections were then cut at 10 or 20 μm on a cryostat (CM1850; Leica). Frozen sections were processed for immunohistochemistry with antibodies against myeloperoxidase (MPO) (1:100; Dako) and ED-1 (1:1000; Serotec) as previously described (23). For quantification of MPO or ED-1 intensity, serial transverse sections (20-μm thickness) were collected every 100-μm section rostral and caudal 3000 μm to the lesion site (total 60 sections). Digital images of MPO- or ED-1-stained tissues were obtained, and we quantified the entire fluorescent intensity of the each transverse section above threshold by using MetaMorph software (Molecular Devices) and averaged. The threshold value was at least 3 times the background, and the background was quantified and normalized to the primary antibody omitted control. Immunostaining control studies were performed by omission of the primary antibodies, by replacement primary antibodies with nonimmune, control antibody, and by preabsorption with an excess (10 μg/mL) of the respective antigens. Serial sections were also stained for histological analysis with Cresyl violet acetate.

**RNA isolation and quantitative real-time RT-PCR**

Total RNA was isolated from spinal cord segments (10 mm), centered at the lesion site by using TRIzol reagent (Invitrogen) according to the manufacturer’s instruction. cDNA was synthesized from 5 μg of the total RNA using Moloney Murine Leukemia Virus-Reverse Transcriptase (Invitrogen), and quantitative real-time RT-PCR was performed using SYBR Green PCR master mix (Invitrogen) as previous described (24). The primers used for real-time PCR were synthesized by the Genotech, and

<table>
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<th>Sequence</th>
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the sequences and annealing temperature of the primers are presented in Table 1.

**Western blotting**

Total protein was prepared with a lysis buffer containing 50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 10mM Na₂HPO₄, 10mM NaF, 1-μg/mL aprotinin, 10-μg/mL leupeptin, 1mM sodium vanadate, and 1mM phenylmethylsulfonylfluoride as previously described (23). Protein sample (30 μg) was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore). The membranes were blocked in 5% nonfat skim milk or 5% bovine serum albumin in Tris-buffered saline containing Tween 20 (0.1%), and subsequently incubated with antibodies against ED-1 (1:200; Serotec), inducible nitric oxide synthase (iNOS) (1:1000; Transduction Laboratory), cyclooxygenase-2 (COX-2) (1:1000; Cayman Chemical), zona occludens-1 (ZO-1) (1:1000; Invitrogen), occludin (1:1000; Invitrogen), TrpM4 (1:1000; Santa Cruz Biotechnology, Inc), and β-tubulin (1:30 000; Sigma). The primary antibody was detected by horseradish peroxidase-conjugated secondary antibodies (1:30 000; Sigma). The primary antibody was detected by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). Immunoreactive bands were visualized by chemiluminescence using Supersignal body (Jackson ImmunoResearch). The primary antibody was detected by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). Immunoreactive bands were visualized by chemiluminescence using Supersignal body (Jackson ImmunoResearch).

**Gelatin zymography**

The activities of MMP-2 and MMP-9 by gelatin zymography were performed using total protein (50 μg) at 1 day after injury as previously described (23, 25). Total protein (50 μg) was loaded on a Novex 10% zymogram gel (EC61752; Invitrogen) and separated by electrophoresis with 100 V (19 mA) at 4°C for 6 hours. The gel was then incubated with renaturing buffer (2.5% Triton X-100) at room temperature for 30 minutes to restore the gelatinolytic activity of the proteins. After incubation with developing buffer (50mM Tris-HCl [pH 8.5], 0.2M NaCl, 5mM CaCl₂, and 0.02% Brij35) at 37°C for 24 hours, the gel was stained with 0.5% Coomassie blue for 24 hours and then destained with 40% methanol containing 10% acetic acid until appropriate color contrast was achieved. Clear bands on the zymogram were indicative of gelatinase activity. Relative intensity of zymography (relative to sham or Veh) was measured and analyzed by Alphalager software (Alpha Innotech Corp). Background was subtracted from the optical density measurements. Experiments were repeated 3 times, and the values obtained for the relative intensity were subjected to statistical analysis.

**Behavioral test**

To test hindlimb locomotor function, open-field locomotion was evaluated by using the Basso-Beattie-Bresnahan (BBB) locomotion scale as previously described (26, 27).

**Statistical analysis**

Data presented as the mean ± SD values and hemorrhage, Evans blue, and BBB data are presented as the mean ± SEM. Comparisons between Veh and E2-treated groups were made by unpaired Student’s t test. Multiple comparisons between groups were performed one-way ANOVA. Behavioral scores from BBB analysis were analyzed by repeated measured ANOVA (time vs treatment). Tukey’s multiple comparison

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**Table 2. Antibody Table**

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<th>Peptide/Protein Target</th>
<th>Antigen Sequence (If Known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
<th>Species Raised in; Monoclonal or Polyclonal</th>
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<td>iNOS</td>
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was used as post hoc analysis. Statistical significance was accepted with $P < .05$. All statistical analyses were performed by SPSS 15.0 (SPSS Science).

**Results**

**E2 inhibits BSCB disruption and hemorrhage after SCI**

Because E2 has been shown to have a protective effect against a BBB breakdown induced by ischemia or vascular endothelial growth factor (18, 19, 28), we hypothesized that E2 would inhibit BSCB disruption/hemorrhage after SCI. Thus, we evaluated the effect of E2 on BSCB disruption/hemorrhage at 1 day after injury using the Evans blue assay and an intraspinal hemorrhage assay. As shown in Figure 1A, the amount of Evans blue dye extravasation increased after SCI when compared with the sham control, which indicates that SCI elicits BSCB disruption. Furthermore, E2 treatment after injury significantly reduced the amount of Evans blue dye extravasation when compared with the Veh control, indicating that E2 treatment attenuated BSCB disruption after SCI ($Veh, 44.8 \pm 3.6$ vs $E2, 19.5 \pm 3.2$; $P < .05$) (Figure 1B). The hemorrhage assay also showed that the amount of intraspinal blood increased after injury but was significantly reduced by E2 treatment when compared with the Veh control ($Veh, 2.3 \pm 0.05$ vs $E2, 1.1 \pm 0.02$; $P < .05$) (Figure 1, C and D). These results indicate that E2 effectively prevents hemorrhage after SCI. In addition, we examined the effect of delayed administration of E2 for clinical relevance. As shown in Figure 1E, BSCB permeability was significantly reduced...
by delayed E2 treatment when the drug was treated at 1 and 2 hours after SCI, whereas significant inhibitory effect of E2 on Evans blue extravasation was not observed in 6-hour-delayed injected group (1-h delay: Veh, 42.3 ± 2.4; E2, 23.5 ± 3.4; 2-h delay: Veh, 43.2 ± 3.1; E2, 32.1 ± 2.7; 6-h delay: Veh, 42.7 ± 2.7; E2 6 h, 39.4 ± 4.5; P < .05).

It is known that the tight junctions (TJs) in the endothelial cells of the blood vessels maintain BBB integrity (Zlokovic [2]). The loss or degradation of TJ proteins has been implicated to mediate the hyperpermeability of BSCB after SCI (23, 25). Thus, we next examined the effect of E2 on the expression levels of the TJ proteins ZO-1 and occludin. First, we examined the effect of E2 on the mRNA level of occludin and ZO-1 using quantitative real-time RT-PCR. As shown Figure 1F, the mRNA level of occludin was decreased at 4 hours after injury and then increased to normal level within 1 day. However, the mRNA level of ZO-1 did not change after SCI and by E2 treatment. We previously showed that the antibodies against ZO-1 and occludin showed specific immunoreactivity at the expected molecular weights for the proteins (65 kDa for occludin, 220 kDa for ZO-1), that the level of ZO-1 and occludin decreased, and that the decrease in ZO-1 and occludin expression was especially prominent at 1 day and 7 days after injury, respectively (25). As shown in Figure 1, G and H, the decrease of the levels of ZO-1 at 1 day and occludin at 7 days after SCI was significantly attenuated by E2 treatment as compared with Veh control, indicating that E2 prevents the loss of TJ proteins after injury (ZO-1; Veh, 0.4 ± 0.06 vs E2, 0.7 ± 0.05; occludin; Veh, 0.3 ± 0.04 vs E2, 0.6 ± 0.05; P < .05).

E2 inhibits MMP-9 expression and activation after SCI

Our recent report shows that the excessive proteolytic activity of MMPs, such as MMP-2 and MMP-9, results in BSCB disruption after SCI (23, 25). Because E2 treatment reduced the BSCB disruption/hemorrhage after SCI (Figure 1), we predicted that E2 would inhibit the expression and activity of MMP-2 and MMP-9 after injury. Rats subjected to contusive SCI at T9 level were killed at 4 hours, 8 hours, and 1 day after injury, and total RNA and protein extracts from spinal cord (10 mm), including the lesion epicenter, were prepared. As shown in Figure 2, A and B, the levels of MMP-2 and MMP-9 mRNA increased after injury compared with the sham control. However, SCI-induced increase in MMP-9 mRNA expression was significantly inhibited by E2 treatment at 8 hours and 1 day after injury compared with the Veh control, but MMP-2 mRNA expression appeared to be unaffected by E2 treatment (Figure 2, A and B). Using gelatin zymography, an increase in the activity of MMP-9 was also observed at 1 day after SCI (active MMP-9) (Figure 2D), but the active MMP-2 band was not detected as previous reports (4, 25). Furthermore, the increase in MMP-9 activity after SCI was significantly inhibited by E2 compared with the Veh control (Figure 2E) (MMP-9; Veh, 4.5 ± 0.2 vs E2, 2.3 ± 0.3; P < .05).

E2 inhibits SUR1 and TrpM4 expression after SCI

It is known that both SUR1 and TrpM4 are up-regulated in the endothelial cells of blood vessels after SCI and mediate progressive hemorrhagic necrosis (6, 9). To investigate whether E2 inhibits SCI-induced hemorrhage, we examined the effect of E2 on SUR1/TrpM4 expression using quantitative real-time RT-PCR and Western blot analysis at 1 hour, 4 hour, 8 hours, and 1 day after injury. As shown in Figure 3A, the mRNA expression of Abcc8 increased at 1 and 4 hours after
injury, and the expression of Abcc8 was significantly inhibited by E2 treatment at 1 and 4 hours after injury. In addition, the mRNA expression of TrpM4 also increased at 8 hours after SCI, and E2 significantly reduced the mRNA level of TrpM4 at 8 hours after injury (Figure 3B). Furthermore, the protein levels of SUR1 and TrpM4 were also reduced by E2 treatment after injury compared with the Veh controls (SUR1; Veh, 8.2 ± 0.7 vs E2, 4.3 ± 0.45; P < .05 vs Veh; TrpM4; Veh, 9.1 ± 0.68 vs E2, 3.8 ± 0.7). The Western blot analysis also revealed that the SCI-induced increase in the ED-1 level in the injured spinal cord lysates was significantly reduced by E2 treatment at 5 days after injury compared with the Veh control (Figure 4C) (Veh, 1.0 ± 0.02 vs E2, 0.4 ± 0.04; P < .05). These findings suggest that E2 inhibits blood cell infiltration by preventing BSCB disruption and hemorrhage after SCI.

E2 inhibits the expression of inflammatory mediators and chemokines after SCI

BSCB disruption and hemorrhage followed by blood cell infiltration after SCI are known to mediate inflammation and initiate secondary injury cascades (23, 25, 29). Infiltrated cells produce inflammatory mediators such as IL-1β, TNF-α, COX-2, and iNOS (29). In addition, the early increase in the expression of chemokines, such as Growth-regulated oncogene (Gro)-α (chemokine (C-X-C motif) ligand [CXCL-1]), MCP-1 (monocyte chemoattractant protein; CCL-2), macrophage inflammatory protein (MIP)-1α (CCL-3), MIP-1β (CCL-4), and MIP-2α (CXCL-2), after SCI is known to induce the infiltration of blood cells such as neutrophils and macrophages, which thereby facilitates inflammatory responses (29, 31–34). Therefore, we postulated that the expression of inflammatory mediators and chemokines would be inhibited by E2 treatment after injury. Using quantitative real-time RT-PCR, the mRNA levels of TNF-α, IL-1β, iNOS, and COX-2 were shown to increase at 2 hours, 6 hours, and 1 day after injury, and these increases were significantly inhibited by E2 (Figure 5A). Western blot analysis also showed that the protein levels of iNOS and COX-2 at 1 day after injury were significantly reduced by E2 compared with the Veh control (Figure 5, B and C). In addition, E2 treatment also significantly inhibited the increases in the mRNA levels of MCP-1, MIP-1α, MIP-1β, Gro-α, and MIP-2α observed at 4 hours, 8 hours, and 1 day after injury (Figure 5D).
The inhibitory effects of E2 on the SCI-induced up-regulation of MMP-9, SUR1, and TrpM4 are mediated by ER.

To determine whether the effects of E2 on SUR1, TrpM4, and MMP-9 activation after SCI are mediated by ER, we used ICI 182780, the ER antagonist. It has been shown that ER-α and ER-β have a 7- and a 3-fold greater affinity, respectively, for ICI 182780 than E2 (35). After SCI, ICI 182780 (3 mg/kg) was administered by ip injection before E2 treatment, the expression of TrpM4, SUR1, and MMP-9 was determined, and the gelatinase activity was measured. As shown in Figure 6, A and B, ICI 182780 treatment significantly attenuated the inhibitory effect of E2 on Abcc8 at 1 h, TrpM4 at 8 h, and MMP-9 at 1 d mRNA expression after SCI (Figure 6, A and B). Western blot analysis also showed that the down-regulation in both SUR1 at 2 h and TrpM4 at 8 h protein expression by E2 after injury was also attenuated by ICI 182780 treatment (Figure 6, C and D). Furthermore, the inhibition of MMP-9 activity by E2 at 1 day after injury was also prevented by the ICI 182780 treatment (Figure 6, E and F). These findings indicate that the inhibitory effects of E2 on the expression of SUR1 and TrpM4 and the expression and activation of MMP-9 are mediated by ER.

Inhibition of BSCB disruption/hemorrhage by E2 is mediated by ER

To further determine whether the inhibitory effects of E2 on the SCI-induced up-regulation in MMP-9, SUR1, and TrpM4 expression are dependent on the ER, we next examined the effect of ICI 182780 on BSCB disruption and hemorrhage at 1 day after injury. As shown in Figure 7, the SCI-induced increases in the amount of Evans blue dye and blood were inhibited by E2, and these effects were significantly inhibited by ICI 182780 treatment after injury. These results suggest that the inhibition of SCI-induced BSCB disruption and hemorrhage by E2 is mediated by ER-α and/or ER-β. Finally, injection of ICI 182780 before E2 treatment significantly abolished the beneficial effect of E2 on functional recovery at 28–35 days after injury (BBB scores at 35 d: Veh, 9.2 ± 0.7; E2, 12.3 ± 0.56; ICI 182780 + E2, 10.3 ± 0.6; P < .05) (Figure 7C).

Discussion

Previously, we reported that the systemic administration of E2 reduces apoptotic cell death and improves functional recovery after a traumatic SCI by up-regulating Bcl-2 expression through phosphoinositide-3-kinase/Akt-dependent cAMP response element-binding protein activation (15, 16). Additionally, we also showed that E2 reduces apoptotic cell death of oligodendrocytes by inhibiting Ras homolog gene family, member A-c-Jun N-terminal kinases 3 activation after SCI (17). However, the mechanisms underlying the neuroprotective effects of E2 have not been fully elucidated. In this study, we demonstrate that E2 inhibits BSCB breakdown and hemorrhage and the subsequent inflammation by down-regulating the expression of SUR1 and TrpM4 and the expression and activation of MMP-9 after SCI. Additionally, we also show that the effect of E2 is mediated by ER-α and/or ER-β. Thus, our study provides evidence that suggests the neuropro-
The protective effect of E2 is, in part, mediated by inhibiting BSCB disruption/hemorrhage after SCI. It is well known that an up-regulation in MMP-9 mediates BSCB disruption by degrading the basal components of TJ proteins, which thereby facilitates the infiltration of immune cells and initiates SCI-induced secondary damage (4). Recently, we reported that the prevention of BSCB disruption by valproic acid and fluoxetine attenuates apoptotic cell death, which is followed by functional recovery after SCI, by inhibiting the up-regulation of MMP-9 and the degradation of TJ proteins (23, 25). Thus, the attenuation of BSCB disruption by inhibiting excessive MMP activity should be considered as a potential therapeutic target to reduce secondary damage after SCI. In this study, we show that E2 treatment significantly decreased the expression and gelatinase activity of MMP-9 at 1 day after SCI (see Figure 2), which thereby attenuated BSCB disruption (see Figure 1, A and B) and that these events were mediated by ER (see Figure 7A). The report by Liu et al (36) showed that E2 protects the BBB by inhibiting MMP-2 and MMP-9 expression and activation in cerebral ischemia. In this study, however, E2 did not inhibit MMP-2 expression and activation after SCI (see Figure 2), even though MMP-2 is up-regulated after SCI. Thus, these results indicate that E2 protects against BSCB disruption after SCI by only inhibiting MMP-9. It has been reported that MMP-9 activity is increased at 12–24 hours after injury; this time frame corresponds with the maximal period of BSCB disruption after SCI. MMP-2 activity, however, is known to increase at a later time (5–7 d) after SCI, and its activity level then remains elevated for weeks. Some reports have shown that MMP-2 facilitates wound healing events and promotes functional recovery after SCI (37, 38). Therefore, the effect of E2 on the activation of MMP-2 within this later time frame after SCI needs to be investigated in a future study. Nevertheless, we cannot exclude the potential involvement of MMP-2 in BSCB disruption after SCI. Recently, we also found and reported that MMP-3 is up-regulated and involved in BSCB disruption after SCI (39). Thus, we will examine the effect of E2 on MMP-3 after SCI in a future study.

TJ proteins, such as occludin and ZO-1, are known to play a critical role in BBB/BSCB integrity (2, 25, 40). Furthermore, it is known that transient focal cerebral isch-
emia-induced BBB disruption is attenuated in MMP-9 knockout mice because of a reduction in ZO-1 degradation compared with wild-type mice (8). As shown in Figure 1, E–H, E2 treatment significantly inhibited the degradation of both occludin and ZO-1 after SCI. Thus, these results suggest that the inhibition of BSCB disruption by E2 after SCI may be mediated by inhibiting MMP-9 activity, which thereby reduces the degradation of TJ proteins.

A small hemorrhagic lesion near the lesion site is observed at an early stage (10–15 min) after SCI and then petechial hemorrhages appear in more distant rostrocaudal tissues at 3–24 hours, resulting in hemorrhagic necrosis (41, 42). The molecular mechanisms underlying progressive hemorrhagic necrosis are known to be mediated by SCI-induced SUR1 and TrpM4 expression in the capillary endothelium (6, 9, 10). In this study, the expression levels of SUR1 and TrpM4 were dramatically up-regulated at an early stage after SCI, which are consistent with previously reported findings. Furthermore, E2 treatment significantly inhibited the up-regulation in SUR1 and TrpM4 expression (see Figure 3). These findings suggest that E2 treatment significantly attenuated hemorrhage after SCI by inhibiting the up-regulation in SUR1 and TrpM4 expression.

Endothelial dysfunction is thought to play an important role in the risk and consequences of hemorrhage after stroke and traumatic SCI (43). It is also known that ER-α and ER-β are expressed in cerebral endothelium (44–46). In addition, we have observed that E2 treatment attenuates TJ breakdown in endothelial cells upon oxygen and glucose deprivation/reperfusion injury in our preliminary study (data not shown). Furthermore, some reports have shown that E2 protects cerebral endothelial cell viability via the ER by increasing mitochondrial efficiency and reducing oxidative stress after ischemic insult in vitro and in vivo (44, 47). Consistent with these findings, our results demonstrate that ICI 182780 treatment significantly blocked the beneficial effects of E2 on BSCB disruption/hemorrhage and locomotor function after SCI (see Figure 7C). In addition, it has been reported that TrpM4, SUR1, and MMP-9 are expressed in the capillary endothelium and that their expression increases after SCI (9, 25). Thus, these results indicate that both the antihemorrhagic and antigelatinolytic effects of E2 may be mediated by ER by inhibiting the expression and activation of TrpM4, SUR1, and MMP-9 in the capillary endothelium, even though we did not examine ER expression in the blood vessels of spinal cord in this study.

It has been known that rodent models show gender differences in functional recovery after SCI, although there is a controversy on gender differences in SCI patients. The improved locomotor function in female mice after SCI may be probably due to beneficial effects of sex hormone.
including estrogen as compared with male mice (48). In fact, beneficial effects of E2 was also observed in brain injury model (49).

Although liganded ER activates directly its target genes, liganded ER also represses gene expression via different mechanisms. For example, E2 treatment inhibits IL-6 gene expression through association of ER, nuclear factor-kappa B, and CEBP on the IL-6 gene promoter (50). Similarly, liganded ER-α interacts with activating protein-1 (AP-1), and this complex transrepresses MMP-1 gene expression (51). Liganded ER-α further exhibits transrepression of aryl hydrocarbon receptor-dependent gene regulation via ER-α interaction with aryl hydrocarbon receptor (52). As ER-α, E2-bound ER-β inhibits MCP-1 gene expression by inhibiting Sp1 and AP-1 transcriptional activities in keratinocytes (53). Recently, it has been reported that liganded ER-β interacts with C-terminal-binding protein corepressor on the AP-1-dependent gene promoters, leading to inflammatory gene repression in microglia and astrocytes (54). Interestingly, Abcc8 gene promoter contains specificity protein-1 and nuclear factor-kappa B binding sites that could be regulated negatively by E2 (55, 56). It is also pointed out that our present study did not elucidate which ER (ER-α or ER-β) mediates the protective effect of E2 on BSCB disruption and functional improvement after SCI. Recent reports showed the function of ER-α and ER-β on BBB disruption in brain injury models. For example, Asl et al (57) showed that the neuroprotective effects of E2 on brain edema, BBB permeability, and neurological scores are mediated through both ER-α and ER-β after traumatic brain injury in rat. In addition, ER-β also contributed to the reduction of vasogetic edema caused by BBB breakdown via the inhibition of hypoxia-inducible factor-1α and vascular endothelial growth factor after ischemic stroke (58). Therefore, we suspect that both ER-α and ER-β mediate the neuroprotective effect on SCI, although there is no systematic examination of the function of ERs using knockout mice or agonist after SCI.

It is well known that the neuroprotective effects of E2 are mediated by diverse mechanisms, such as genomic, nongenomic, receptor, and nonreceptor (59, 60). Thus, further studies elucidating the mechanisms underlying the effect of E2 on the viability of capillary endothelial cell through an increase in mitochondrial efficiency and a reduction in oxidative stress after SCI will be examined, even though the effect of E2 on BSCB disruption and hemorrhage after SCI was ER dependent. In addition, the role ER-mediated signaling plays in the inhibition of SUR1, TrpM4, and MMP-9 expression by E2 will be examined in a future study.

In summary, our findings show that the neuroprotective effect of E2 is mediated, in part, by inhibiting the up-regulation of SUR1, TrpM4, and MMP-9 expression by E2 will be examined in a future study.

Figure 7. ICI 182780 treatment blocks the protective effects of E2 on BSCB disruption/hemorrhage and functional improvement after SCI. Injured rats were administered ICI 182780 (3 mg/kg) via ip injection before E2 treatment, and BSCB disruption and hemorrhage were assayed as described in Materials and Methods (n = 5/group). Quantification of the Evans blue extravasation (A) and spectrophotometric quantification of the amount of intraspinal blood in homogenates (B). Note that the inhibitory effects of E2 on BSCB disruption and hemorrhage were reversed by the ER antagonist. The value is presented as an amount of dye (μg)/tissue weight (g). C, BBB locomotor scale. Injured rats were administered ICI 182780 (3 mg/kg) via ip injection before E2 treatment, and functional recovery by BBB test was performed as described in Materials and Methods (n = 15/group). Note that ICI 182780 treatment alleviated the beneficial effect of E2 on BBB locomotor score. Data are presented as mean ± SEM. *, P < .05; **, P < .01 vs Veh; #, P < .05 vs E2.
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