Premarin stimulates estrogen receptor-α to protect against traumatic brain injury in male rats

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Objectives: To establish mechanisms of neuroprotective actions induced by Premarin (an estrogen sulfate) during traumatic brain injury.

Design: Chi Mei Medical Center research laboratory.

Subjects: Male Sprague-Dawley rats 244 to 268 g.

Interventions: Anesthetized rats, immediately after the onset of fluid percussion injury, were divided into three major groups and given the vehicle solution (1 mL/kg of body weight), Premarin (1 mg/kg of body weight), or Premarin (1 mg/kg of body weight) plus the nonselective estrogen receptor-α antagonist ICI 182, 780 (0.25 mg/kg of body weight) intravenously and immediately after fluid percussion injury.

Measurements and Main Results: Premarin, in addition to inducing pharmacologic levels of estradiol, causes attenuation of fluid percussion injury-induced cerebral infarction and motor and cognitive function deficits. Fluid percussion injury-induced apoptosis (e.g., increased numbers of both terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive and caspase-3-positive cells) as well as activated inflammation (e.g., increased levels of tumor necrosis factor-α) was also significantly Premarin-reduced. In peri-ischemic areas of hippocampus, both angiogenesis (e.g., increased numbers of both 5-bromodeoxyuridine-positive endothelial and vascular endothelial growth factor-positive cells) and neurogenesis (e.g., increased numbers of both 5-bromodeoxyuridine/neuronal-specific nuclear protein double-positive and glial cell line-derived neurotrophic factor-positive cells) were Premarin therapy-promoted. In estrogen receptor-α blockade rats, Premarin therapy had less or no effect on fluid percussion injury-induced behavioral deficits, cerebral infarction and apoptosis, and activated inflammation. Furthermore, Premarin-induced angiogenesis and neurogenesis were estrogen receptor-α blockade-reduced.

Conclusions: Our results indicate that pharmacologic levels of Premarin therapy-induced estradiol protect against cortical and hippocampal programmed cell death after fluid percussion injury through mechanisms stimulating estrogen receptor-α in the male rats. (Crit Care Med 2009; 37:3097–3106)

Key Words: traumatic brain injury; conjugated; estrogen; inflammation; angiogenesis; neurogenesis

Premarin (Wyeth-Ayerst Laboratories, Rouse Point, NY) is a widely used compound for estrogen replacement therapy for uterine bleeding in postmenopausal women. The compound is a pH-balanced mixture of conjugated estrogens (Wyeth-Ayerst Laboratories, Rouse Point, NY) occurring as sodium salts of water-soluble estrogen sulfate (including estrone, equilenin, 17α-dihydroequilenin, 17α-estradiol, equilenin, and 17α-dihydroequilenin) with lactose, sodium citrate, and simethicone binders (1). Premarin, like 17β-estradiol, has significant systemic, coronary, and uterine vascular effects (2). A more recent report has shown that Premarin protects against traumatic brain injury by reducing apoptosis in rats after a contusion of the parietal cortex (3). A more recent report has demonstrated that Premarin can act through estrogen receptor (ER-α) and β to protect hippocampal neurons against global ischemia-induced cell death (4). This raises the possibility that Premarin may act through ER to exert their neuroprotection during traumatic brain injury.

The purposes of the presented study were: 1) to determine whether the neuroprotective effects of Premarin in a traumatic brain injury model, which produces selective cerebral infarction and apoptosis as well as motor and cognitive dysfunction, can be reduced by a nonselective ER antagonist, ICI 182, 780 (4); and 2) to identify whether the enhanced neurogenesis and angiogenesis and the reduced brain inflammation after Premarin therapy can be attenuated by ICI 182, 780 in this model. Resultantly, the broad-spectrum ER-α antagonist ICI 182, 780 abolished the neuroprotective effects of Premarin in male rats subjected to traumatic brain injury by reducing neurogenesis, angiogenesis, and brain inflammation.

METHODS

Animals. Male Sprague-Dawley rats (weight, 256 ± 12 g) were obtained from the Animal Resource Center of the National Science Council of Republic of China (Taipei, Taiwan). Support was provided by the Department of Obstetrics and Gynecology (SHC), Taipei Medical University, Taipei, Taiwan; Stem Cell Research Center (HKC, WCC), National Cheng Kung University School of Medicine, Tainan, Taiwan; Department of Obstetrics and Gynecology (FMC), National Cheng Kung University School of Medicine, Tainan, Taiwan; Department of Obstetrics and Gynecology (FMC), National Cheng Kung University School of Medicine, Tainan, Taiwan; Department of Obstetrics and Gynecology (SHC), the Department of Neurology (CYC), and the Department of Medical Research (MTL, JJW), Chi Mei Medical Center research laboratory.

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Taiwan). The animals were housed four in a group at an ambient temperature of 22 ± 1°C with a 12-hr light–dark cycle. Pelleted rat chow and tap water were available ad libitum. All protocols were approved by the Animal Ethics Committee of the Chi Mei Medical Center (Tainan, Taiwan) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Guidelines for the Animals Welfare Act to minimize discomfort in the animals during surgery and in the recovery period.

Surgery. Animals were anesthetized with sodium pentobarbital (25 mg/kg, intraperitoneally; Sigma Chemical, St. Louis, MO) and a mixture containing ketamine (4.4 mg/kg, intramuscularly; Nankuang Pharmaceutical, Taipei, Taiwan), atropine (0.0263 mg/kg, intramuscularly; Sintong Chemical, Ind., Taoyuan, Taiwan), and xylazine (6.77 mg/kg, intramuscularly; Bayer, Lauerkusen, Germany). Both the femoral artery and vein on the right side were cannulated with PE50 polyethylene tubing for monitoring blood pressure and analyzing blood gas. After cannulation, the wound was sutured and animals were turned prone. The animals were placed in a stereotaxic frame, and the scalp was incised sagittally. The animals were subjected to a lateral fluid percussion injury (FPI) (5). After the scalp was incised, a 4.8-mm circular craniotomy was performed midway between lambda and bregma 3.0 mm to the right of the central suture. A modified Luer-lock connector (trauma cannula), 2.6 mm inner diameter, was secured into the craniotomy with cyanacrylate adhesive and dental acrylic. A moderate FPI (2.2 atm) was produced by rapidly injecting a small volume of saline into the closed cranial cavity with a fluid percussion device (VCU Biochemical Engineering, Richmond, VA). Immediately after the traumatic event, the animals displayed brief respiratory arrests and limb convulsions lasting for 40 to 70 secs. In addition, hypertension, tachycardia, intracranial hypertension, cerebral ischemia, and hypoxia were noted for certain time periods. The animal was removed from the device, the acrylic removed, and the incision sutured. Each injured and sham-injured animal for the fluid percussion model was closely evaluated immediately after FPI for behavioral recovery.

Experimental Procedures. In experiment 1, an intravenous dose of Premarin (1 mg/kg) or saline was randomly administered immediately after FPI (n = 16), and their effects on mean arterial pressure, heart rate, intracranial pressure, cerebral perfusion pressure, core temperature, the hippocampus level of NO2−, glutamate, glycerol, the lactate-to-pyruvate ratio, and the plasma levels of estradiol were assessed immediately during FPI for 120 mins. In experiment 2, an intravenous dose of Premarin (1 mg/kg), ICI 182,780 (0.25 mg/kg), or saline was randomly administered immediately after FPI and their effect on the maximal angle animals could cling to an inclined plane and Morris Water Maze performance were assessed 3 days after FPI.

In experiment 3, an intravenous dose of Premarin (1 mg/kg), ICI 182,780 (0.25 mg/kg), or saline was randomly administered immediately after FPI and their effect on cerebral infarction zone was assessed 7 days after FPI.

In experiment 4, an intravenous dose of Premarin (1 mg/kg), ICI 182,780 (0.25 mg/kg), or saline was randomly administered immediately after FPI and their effects on the amounts of terminal deoxynucleotidyl transferase diUTP nick-end labeling-positive cells and caspase-3-positive cells in the peri-ischemic area were assessed 3 days after FPI.

In experiment 5, an intravenous dose of Premarin (1 mg/kg), ICI 182,780 (0.25 mg/kg), or saline was randomly administered immediately after FPI, and their effects on the amounts of antineuronal-specific nuclear protein (NeuN)-positive, bromodeoxyuridine (BrdU)-positive, vascular endothelial growth factor (VEGF)-positive, and glial cell lines derived neurotrophic factor (GDNF)-positive BrdU/NeuN double-positive, and BrdU-positive endothelial cells in the peri-ischemic area were assessed 3 days after FPI.

Table 1. Plasma levels of estradiol (E2) for different groups of rats

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Plasma E2, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham controls</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>(FPI + saline)-treated rats</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>(FPI + Premarin)-treated rats</td>
<td>2269 ± 338*</td>
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</table>

*p < .05 in comparison to group 2. Vehicle or Premarin (5 mg/kg, intravenously) was administered immediately after the onset of fluid percussion injury (FPI), and the blood sampling was obtained for E2 assay 20 mins after vehicle or Premarin injection. The data are mean ± SEM of eight rats per group.

Figure 1. Premarin decreases the fluid percussion injury (FPI)-induced intracranial hypertension and cerebral hyperperfusion. The (FPI + saline)-treated group (○; n = 8) had a significance increase of intracranial pressure (ICP) but a significant decrease of cerebral perfusion pressure (CPP) at 10 to 120 mins after FPI (p < .01) compared with the sham controls (●; n = 8). The (FPI + Premarin)-treated group (ateurs; n = 8) showed a significant decrease of ICP but a significant increase of CPP at 10 to 120 mins after FPI (p < .01) compared with the (FPI + saline)-treated group (○; n = 8). The dashed line denotes the onset of FPI. MAP, mean arterial pressure.
In experiment 6, an intravenous dose of Premarin (1 mg/kg) or saline was randomly administered immediately after FPI, and their effects on both the serum and hippocampal levels of interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) were assessed 3 days after traumatic brain injury.

**Monitoring of Physiological Parameters.** For the measurement of intracranial pressure, the animals were positioned in a stereotactic apparatus (Kopf Instruments, Tujunga, CA) with the nose bar positioned 3.3 mm below the horizontal line. After a midline incision in the skull, a dialysis probe (4 mm in length, CMA/2; Carnegie Medicine, Stockholm, Sweden) was inserted. The microdialysis probe was stereotaxically and obliquely (anterior 4.3 mm) implanted into the right hippocampus according to the atlas and coordinates of Paxinos and Watson (6): A, interaural, 7.7 mm; L, 2.0 mm from the midline; and H, 3.5 mm from the top of the skull. All recordings were made on a four-channel Gould polygraph. Core temperature was monitored continuously by a thermocouple, and mean arterial pressure and heart rate were continuously pressure transducer-monitored.

The animal's head was mounted in a stereotactic apparatus (David Kopf Instruments, Tujunga, CA) with the nose bar positioned 3.3 mm below the horizontal line. After a midline incision in the skull, a dialysis probe (4 mm in length, CMA/2; Carnegie Medicine, Stockholm, Sweden) was inserted. The microdialysis probe was stereotaxically and obliquely (anterior 4.3 mm) implanted into the right hippocampus according to the atlas and coordinates of Paxinos and Watson (6): P, 8 mm; R, 3 mm; H, 5 mm. According to the methods described previously (7, 8), the microdialysis was perfused at 2.0 μL/min and the dialysates were sampled in microvials. The dialysates were collected every 20 mins in a CMA/140 fraction collector (Carnegie Medicine). Aliquots of dialysates (5 μL) were injected onto a CMA 600 microdialysis analyzer (Carnegie Medicine) for measurement of lactate, glyc erol, pyruvate, and glutamate. The NO2 concentrations in the dialysates were measured with the Eicom ENO-20 NO2 analysis system (Eicom, Kyoto, Japan) (9). Only experiments in which the hippocampal localization of the microdialysis probes was confirmed histologically were included in the results.

**Inclined Plane.** The inclined plane was used to measure limb strength. The animals were placed, facing right and then left, perpendicular to the slope of a 20 × 20-cm rubber ribbed surface of an inclined plane starting at an angle of 55° (10, 11). The angle was increased or decreased in 5° increments to determine the maximal angle an animal could hold to the plane. The data for each day were calculated as 2 mm (thickness of the slice) × [sum of the infarction area in all brain slices (mm²)] (12).

**Cerebral Infarction Assay.** The triphenyltetrizolium chloride staining procedures followed those described elsewhere (12). All the animals were killed at day 3 after FPI. Under deep anesthesia (sodium pentobarbital, 100 mg/kg, intraperitoneally), the animals were perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 mins, and sliced into 2.0-mm sections with a tissue slice. The brain slices were incubated in 2% triphenyltetrizolium chloride dissolved in phosphate buffered saline (PBS) for 30 mins at 37°C and then transferred to 5% formaldehyde solution for fixation. The volume of infarction, as revealed by negative triphenyltetrizolium chloride stains indicating dehydrogenase-deficient tissue, was measured in each slice and summed using computerized planimetry (PC-based Image Tools Software; Media Cybernetics, Inc., Bethesda, MD). The volume of infarction was calculated as 2 mm (thickness of the slice) × [sum of the infarction area in all brain slices (mm²)] (12).

**Assessment of Cognitive Function.** Cognitive testing for testing (spatial learning) was assessed using the hidden platform version of the Morris Water Maze (13). The Morris Water Maze apparatus (Muromachi Kikai Co., Ltd, Tokyo, Japan) consisted of a large, gray circular pool (147 cm diameter, 45 cm height) and a 10-cm-diameter Plexiglas transparent platform submerged 1 cm below the water surface. The rats were trained to locate a hidden, submerged platform using constant extramaze visual information. During experiments, the platform remained in a constant location in one quadrant. The rats were gently placed in the water facing the wall at one of four randomly chosen locations separated by 90°. The latency to find the hidden platform within an 180-sec maximal time was recorded using a computerized video tracking system (Muromachi Kikai Co, Ltd, Tokyo, Japan). Trials were conducted at 3 days postinjury.

**Figure 2.** Premarin decreased the fluid percussion injury (FPI)-induced increased levels of cellular ischemia and damage markers in the hippocampus. The (FPI + saline)-treated group (●; n = 8) showed a significant increase of hippocampal levels of NO2, glutamate, glycerol, and lactate/pyruvate ratio at 10 to 120s min after FPI (●; n = 8). The (FPI + Premarin)-treated group (●; n = 8) had a significant decrease of NO2, glutamate, glycerol, and lactate/pyruvate ratio in the hippocampus compared with the (FPI + saline)-treated group (●; n = 8). Tco, core temperature; NO2, nitric oxide metabolite.
Bromodeoxyuridine Labeling. To evaluate the proliferation of cells, BrdU (Roche Diagnostics, Indianapolis, IN; 50 mg/kg) dissolved in PBS was administered once daily intraperitoneally for 3 days after FPI. The rats were killed 3 days later.

Immunohistochemistry. Adjacent 50-µm sections, corresponding to coronal coordinates 0.20 mm to 0.70 mm anterior to the bregma, were incubated in 2 mol/L HCl for 30 mins, rinsed in 0.1 mol/L boric acid (pH 8.5) for 3 mins at room temperature, and then incubated with primary antibodies in PBS containing 0.5% normal bovine serum at 4°C overnight. After being washed in PBS, the sections were incubated with secondary antibodies for 1 hr at room temperature. The following antibodies were used in this study: rat monoclonal anti-BrdU antibody (1:10; Oxford Biotechnology, Oxfordshire, UK), mouse anti-VEGF, anti-GDNF, or anti-NeuN antibody (1:200; Vector Laboratories, Burlingame, CA) were used in the same manner instead of other primary antibodies and visualized using fluorescein-labeled streptavidin (1:200; Vector Laboratories) for endothelial cells, biotinylated Lycopersicon esculentum (Tomato) lectin (1:200; Vector Laboratories) for microglia, and rat monoclonal anti-BrdU antibody (1:10; Oxoid Biotechnology, Oxfordshire, UK), mouse anti-VEGF, anti-GDNF, or anti-NeuN antibody (1:200; Vector Laboratories) for microglia. The sections were then washed in PBS/0.1% TX-100 for 60 mins. They were mounted with antifade mounting media. The number of labeled cells was calculated in five coronal sections from each rat and expressed as the mean number of cells per section. For negative control sections, all the procedures were performed in the same manner without the primary antibodies. Co-labeling all tissue with DAPI (a non-specific nuclear marker) and expressing the immunohistochemically positive cells as a ratio to all DAPI-positive cells were performed. All cell counting was done in a blinded manner to avoid all bias.

Radioimmunoassay. Plasma estradiol concentrations were determined using the double-antibody radioimmunoassay kits (rat estradiol radioimmunoassay kit; Immunootech, Marseille, France). One hundred (estradiol) microliter plasma samples were assayed in duplicate. The cross-reactivity of the radioimmunoassay for estradiol was 100%. The test values were determined by interpolation from the standard curves. The lowest detectable levels of estradiol were 20 ng/mL.

Terminal Deoxynucleotidyl-Transferase-Mediated dUTP-Biotin Nick End-Labeling Assay. The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using the same brain tissues used in histologic verification. The color was developed using 3,3-diamino-benzidine tetra-chloride. The sections were xylene- and ethanol-dehydrated. They were then incubated with 1% H2O2 solution for 20 mins. The sections were incubated in 3% H2O2 solution for 20 mins, rinsed with PBS and then washed with distilled water. They were then reincubated in an antifluorescein antibody conjugated with horseradish peroxidase at room temperature for 30 mins, rewashed, and then visualized using the avidin–biotin–peroxidase complex technique and 0.05% 3,3-diamino-benzidine tetra-chloride (Sigma Chemical) as a chromogen. The numbers of TUNEL-positive cells were pathologist-counted in 30 fields/sections (×200 magnification). The blinding was performed for the pathology grading of the results.

Assay of Cytokines. For the determination of TNF-α and IL-10, the blood or brain (hippocampal formation) samples were taken 3 days after FPI or the equivalent time for the sham-operated rats. The blood samples were allowed to clot for 2 hrs at room temperature and then were centrifuged at 2000 × g for 20 mins at 4°C. The supernatants were collected and stored at −70°C until time of measurement. The brain samples were prepared according to previous reports (14). The concen-

**Figure 3.** Premarin decreased the fluid percussion injury (FPI)-induced motor (A) and cognitive (B) dysfunction. The FPI plus saline-treated group or the FPI plus ICI 182, 780-treated group showed a significant decrease of maximal animal angles but a significant increase of latency to locate the platform in Morris Water Maze at 3 days after FPI compared with the sham controls (*p < .01). The FPI plus Premarin-treated group showed a significant increase of the maximal angle but a significant decrease of latency to reach the platform compared with the FPI plus saline-treated group (p < .01). The FPI plus Premarin plus ICI 182, 780-treated group had a significant decrease of maximal angle but a significant increase of latency to arrive at the platform compared with the FPI plus Premarin group (p < .01). Each column and bar denotes mean ± SD of eight animals per group.
Concentrations of TNF-α and IL-10 in the samples were determined using double-antibody sandwich enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The optical densities were read on a plate reader set at 450 nm for TNF-α and IL-10. The concentrations of TNF-α and IL-10 in the samples were calculated from the standard curve multiplied by the dilution factor and were expressed as pg/mL.

**Statistical Analysis.** The data are presented as mean ± SD. The repeated measures analysis of variance was conducted to test the treatment-by-time interactions and the effect of treatment over time on each score. The Duncan's multiple range test was used for *post hoc* multiple comparisons among means. *p* < .05 was considered evidence of statistical significance.

**RESULTS**

**Premarin Increases Plasma Levels of Estradiol During FPI.** Compared with the sham controls or the vehicle-treated FPI rats, the Premarin-treated FPI rats had significantly higher plasma estradiol levels at 20 mins after the start of FPI (Table 1).

**Premarin Attenuates Cerebrovascular Dysfunction During FPI.** The values for intracranial pressure and hippocampal levels of nitrates, glycerol, glutamate, and lactate-to-pyruvate ratio in vehicle-treated FPI rats were all significantly higher at 20 to 120 mins after the start of FPI than they were for the sham-operated controls. In contrast, the values for cerebral perfusion pressure were significantly lower than those of the sham-operated controls (Figs. 1 and 2). Resuscitation with Premarin (1 mg/kg) immediately after FPI significantly attenuated the FPI-induced intracranial hypertension, cerebral hypoperfusion, and alteration in microdialysis markers of cellular dysfunction (7, 8) in hippocampus.

**Premarin Improves Motor and Cognitive Function During FPI.** At 3 days after FPI, behavioral tests revealed that the vehicle-treated FPI rats had significantly lower performance in both motor and cognitive function tests than they were for sham-operated controls (Fig. 3). The FPI-induced motor and cognitive dysfunction could be significantly reduced by Premarin treatment. Although ICI 182, 780 itself was ineffective on all behavioral tests, it did reverse the beneficial effects exerted by Premarin in dealing with the FPI-induced behavioral dysfunc-

**Premarin Decreases Infarct Area During FPI.** The triphenyltetrazolium chloride-stained sections at 3 days after FPI showed a significant decrease in the infarcted area of the Premarin-treated group (Figs. 4 and 5). Both the cortical and hippocampal infarction areas were distinctly smaller in the Premarin-treated rats than in vehicle-treated ones. However, the beneficial effects of
Figure 6. Premarin decreases the fluid percussion injury (FPI)-induced apoptosis. The FPI plus saline-treated group or the FPI plus ICI 182, 780-treated group showed a significant increase of the numbers of both terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive (A) and caspase-3-positive (B) cells in the ischemic hippocampus at 3 days after FPI compared with the sham controls (*p < .01). The FPI plus Premarin-treated group had a significant decrease of the numbers of both TUNEL-positive and caspase-3-positive cells compared with the FPI plus saline-treated group (#p < .01). The FPI plus ICI 182, 780 plus Premarin-treated group had a significant increase of the numbers of both TUNEL-positive and caspase-3-positive cells in the ischemic hippocampus compared with the traumatic brain injury (TBI) plus saline plus Premarin-treated group (†p < .01). Each column and bar denotes mean ± SD of eight animals per group. (a–e in A and B): representative TUNEL staining and caspase-3 staining at 3 days after FPI, respectively, for a sham control (a), an FPI plus saline-treated rat (b), an FPI plus ICI 182, 780-treated rat (c), an FPI plus Premarin-treated rat (d), and an FPI plus ICI 182, 780 plus Premarin-treated rats (e).
Premarin were significantly lessened by ICI 182, 780 treatment (Fig. 4). In particular, the ischemic hippocampal areas included the dentate gyrus during traumatic brain injury (Fig. 5).

**Premarin Decreases Cerebral Apoptosis During FPI.** Both the TUNEL-stained and caspase-3-stained sections at 3 days after FPI revealed a significant decrease in numbers of both TUNEL-positive and caspase-3-positive cells in the peri-ischemic area of the Premarin-treated group. Although ICI 182, 780 itself was ineffective on numbers of both TUNEL-positive and caspase-3-positive cells in the peri-ischemic area, it did reverse the beneficial effects exerted by Premarin in dealing with the FPI-induced cerebral apoptosis.

**Premarin Promotes Neurogenesis During FPI.** In the peri-ischemic area, BrdU plus NeuN double-positive cells (Fig. 7A) and GDNF-positive cells (Fig. 7B) increased in the Premarin-treated group ($p < .05$) compared with the vehicle-treated group at 3 days after FPI, but the number of both NeuN plus BrdU double-positive and GDNF-positive cells was significantly reduced in the Premarin plus ICI 182, 780-treated group ($p < .05$).

**Premarin Promotes Angiogenesis During FPI.** In the peri-ischemic area, both BrdU-positive endothelial (Fig. 8A) and VEGF-positive (Fig. 8B) cells increased in the Premarin-treated group ($p < .05$) compared with the vehicle-treated group at 3 days after FPI, but the number of both BrdU-positive endothelial and VEGF-positive cells was significantly reduced in the Premarin plus ICI 182, 780-treated group ($p < .05$).

**Premarin Attenuates Systemic and Brain Inflammation During FPI.** Both the serum and brain levels of TNF-α and IL-10 decreased and increased, respectively, in the Premarin-treated group ($p < .05$; Fig. 9), compared with the vehicle-treated group at 3 days after FPI, but the levels of both TNF-α and IL-10 were significantly reduced in the Premarin plus ICI 182, 780-treated group ($p < .05$; Fig. 9).

**DISCUSSION**

Our previous results have demonstrated that the heat tolerance of estrus female rodents is superior to that of male...
rodents (15, 16). On the other hand, the heat tolerance of surgically or chemically ovarioctomized rats is inferior to that of estrus female rats. In addition, estrogen replacement by Premarin in male, estrus female, and surgically or chemically ovarioctomized rodents greatly improves heat tolerance. In the present study, we further demonstrate that Premarin protects male rats from FPI-induced cerebral infarction and functional deficits. The results are supported in part by several experimental studies. For example, estrogen reduces injury after global (17–19) and focal (1, 20) cerebral ischemia, subarachnoid hemorrhage (21), traumatic brain injury (3), and heat-induced cerebral ischemia and injury (15, 16).

It has been documented that low, physiological concentrations of estradiol are able to exert protection against ischemia induced by middle cerebral artery occlusion in the adult rats (22). Protection by low estradiol levels necessitates pretreatment (22, 23) and requires the ER-α (24). Additionally, pharmacologic doses of estradiol may bypass ERs to inhibit NMDA receptors (25) and attenuate oxidative injury (26–28). The neuroprotective effects of Premarin during FPI in the present studies are also ER-α activity-related because Premarin therapy has less or no effect on ER-α blockade rats. Premarin may cause pharmacologic levels of estradiol and attenuate apoptosis in the ischemic area after a FPI by upregulation of bcl-2 (3) as well as the downregulation of both glutamate and nitric oxide metabolites in rats (present results).

In the adult brain, new neurons are continuously generated in both the hippocampus and subventricular zones. The proliferated cells generated from these areas can migrate to and differentiate in the damaged region of the brain (29–31). We found here that most ischemic administration of Premarin promoted generation of proliferated neurons (evidenced by the increased numbers of NeuN plus BrdU double-positive cells) in the ischemic hippocampus 3 days after FPI (Fig. 7). As shown in Figure 5, Premarin improved FPI by augmenting neurogenesis in both cortical and hippocampal regions. In particular, the peri-ischemic hippocampus included the dentate gyrus.

Evidence has accumulated to indicate the neurotrophic effect of estrogen therapy. For example, Toran-Allerand and others (32) demonstrated that estradiol treatment of explant cultures of newborn mouse hypothalamus stimulates extensive neurite outgrowth. Estradiol also increases cell viability, differentiation, neurite outgrowth, and spine density and controls the ability of neurons to extend neurites and to form synaptic connec-

Figure 8. Premarin promotes angiogenesis. The fluid percussion injury (FPI) plus Premarin-treated group showed a significant increase of the numbers of both bromodeoxyuridine (BrdU)-positive endothelial (E) and vascular endothelial growth factor (VEGF)-positive (F) cells in the ischemic hippocampus at 3 days after FPI compared with the sham controls, FPI plus ICI 182, 780-treated group, or FPI plus saline group (\#p < .01). The FPI plus ICI 182, 780 plus Premarin-treated group had a significant decrease of the numbers of both BrdU-positive endothelial and VEGF-positive cells in ischemic hippocampus at 3 days after FPI compared with the (FPI plus saline plus Premarin-treated group (\#p < .01). Each column and bar denotes mean ± so of eight animals per group. Top panels: representative double immunofluorescent staining at 3 days after FPI. The square in A indicates the enlarged double-labeled cells in panels B-D. BrdU-positive cells (red, C) are also positive for endothelin (green B; merged D). Scale bar = 20 μm. (a–e in Fig. 7B): representative VEGF staining at 3 days after FPI, respectively, for a sham control (a), an FPI plus saline-treated rat (b), an FPI plus ICI 182, 780-treated rat (c), an FPI plus Premarin-treated rat (d), and an FPI plus ICI 182, 780 plus Premarin-treated rat (e).
TNF-α and insulin-like growth factor-1, transforming growth factors such as nerve growth are also shown to modulate the synthesis or in neuroblastoma cells (33). Estrogens spines in dissociated neurons in culture

...mune system contributes to neurofilament protein (NFP)-induced activated inflammation. Both FPI plus saline-treated (III) and (FPI plus ICI 182, 780)-treated (III) groups showed a significant increase of serum or brain tumor necrosis factor (TNF-α) at 3 days after FPI compared with the sham controls (II). The FPI plus Premarin-treated group (III) showed a significant decrease of serum or brain TNF-α at 3 days after FPI compared with the FPI plus saline-treated group (III; \( p < .01 \)). The FPI plus Premarin plus ICI 182, 780-treated group (III) had a significant increase of serum or brain TNF-α at 3 days after FPI compared with the FPI plus Premarin-treated group (III; \( p < .01 \)). The FPI plus Premarin plus ICI 182, 780-treated group had a significant increase of serum or brain IL-10 at 3 days after FPI (III) compared with the FPI plus Premarin-treated group (III; \( p < .01 \)).

In summary, we found that Premarin caused pharmacologic levels of estradiol and resulted in attenuating cerebral infarction and functional deficits after a FPI. These findings indicate that pharmacologic levels of estradiol induced by Premarin therapy protect against cortical and hippocampal programmed cell death after FPI through mechanisms stimulating ER-α.

REFERENCES


CONCLUSIONS

In summary, we found that Premarin induced inflammation has been shown to impair neurogenesis (40), whereas blockade of inflammation has been shown to preserve neurogenesis (41, 42) after brain injury. In addition, inflammation in autoimmune encephalomyelitis (43) and brain ischemia (44) can be ER-reduced. The current study further demonstrates that Premarin ameliorates both systemic and brain inflammation during traumatic brain injury by decreasing TNF-α but increasing IL-10 production in both the serum and the brain. In fact, IL-10 has important anti-inflammatory properties through suppression of TNF-α and other proinflammatory cytokines (45).

We need to acknowledge the study limitations, e.g., males only, limited age range, study at one time point (i.e., 3 days only), no dose effect, and demonstrating mechanisms of action only by limited means. Therefore, many more prospective studies are required.

Tensions with their cells through dendritic spines in dissociated neurons in culture or in neuroblastoma cells (33). Estrogens are also shown to modulate the synthesis of growth factors such as nerve growth factor, brain-derived neurotrophic factor, insulin-like growth factor-1, transforming growth factor-β, and related receptors, Trk A and Trk B, in neurons and astroglia (34, 35).

Indeed, as shown in the present results, systemic administration of Premarin, a conjugated estrogen, stimulates production of both VEGF and GDNF and increases the amounts of BrdU-positive endothelial cells in the injured brain. These findings support the hypothesis that Premarin may promote an environment conductive to revascularization of ischemic brain so that neuronal regeneration can proceed. The hypothesis is supported by many investigators. Topical application of GDNF and adenovirus-mediated GDNF gene transfer significantly reduce infarct size in a rat middle cerebral artery ligation model (36, 37). A rich vascular environment, along with generation of VEGF, may enhance subsequent angiogenesis and neurogenesis (38, 39). These findings suggest that Premarin may improve both cognitive and motor function recovery after FPI by enhancing neovessel formation and accelerating endogenous neurogenesis.

Evidence has accumulated to indicate that systemic inflammation contributes to cell death after cerebral ischemia and impairs neurogenesis. For example, endotoxin-induced inflammation has been shown to impair neurogenesis (40), whereas blockade of inflammation has been shown to restore neurogenesis (41, 42) after brain injury. In addition, inflammation in autoimmune encephalomyelitis (43) and brain ischemia (44) can be ER-reduced. The current study further demonstrates that Premarin ameliorates both systemic and brain inflammation during traumatic brain injury by decreasing TNF-α but increasing IL-10 production in both the serum and the brain. In fact, IL-10 has important anti-inflammatory properties through suppression of TNF-α and other proinflammatory cytokines (45).

We need to acknowledge the study limitations, e.g., males only, limited age range, study at one time point (i.e., 3 days only), no dose effect, and demonstrating mechanisms of action only by limited means. Therefore, many more prospective studies are required.

CONCLUSIONS

In summary, we found that Premarin caused pharmacologic levels of estradiol and resulted in attenuating cerebral infarction and functional deficits. It seems that ER-α is associated with the neuroprotection of Premarin because Premarin therapy had less or no effect in ER-α blockade animals. Premarin could act through promotion of both angiogenesis and neurogenesis as well as reduction of systemic inflammation to attenuate cere-


42. Hoehn BD, Palmer TD, Steinberg GK: Neurogenesis in rats after focal cerebral ischemia is enhanced by indomethacin. *Stroke* 2005; 36:2718–2724

