17 β -Estradiol Pretreatment Prevents the Global Ischemic Injury-Induced Decrease of Akt Activation and Bad Phosphorylation in Gerbils

Phil-Ok KOH^{1)*}, Gyeong-Jae CHO²⁾ and Wan-Sung CHOI²⁾

¹⁾Department of Anatomy, College of Veterinary Medicine and Research Institute of Life Science, Gyeongsang National University, 900 Gajwa-dong, Jinju 660-701 and ²⁾Department of Anatomy and Neurobiology, Health Science Institute, College of Medicine, Medical Research Center for Neural Dysfunction, Gyeongsang National University, 92 Chilam-dong, Jinju 660-751, South Korea

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ABSTRACT. Estradiol acts as a neuroprotective factor against ischemic brain injury. This study investigated whether estradiol modulates neuroprotective mechanism through the activation of Akt and its downstream target such as Bad in global ischemic injury. Adult female gerbils were ovariectomized and treated with estradiol prior to ischemic injury. Transient cerebral ischemia was accomplished by bilateral clipping of the common carotid artery for 5 min. Brains were collected on 1, 3, 5 day after injury. In hippocampal CA1 region of non-treated gerbils, most of neuronal cells exhibited pyknotic nuclei and showed the positive reaction of TUNEL staining on 5 day after injury. However, estradiol significantly reduced the neuronal cell death. Potential activation was measured by phosphorylation of Akt at Ser⁴⁷³ and Bad at Ser¹³⁶ using western blot analysis. The levels of pAkt and pBad were significantly decreased in non-treated gerbils on 1–5 day after injury. However, estradiol prevents the global ischemic injury-induced decrease of pAkt and pBad. Our findings suggest that estradiol prevents cell death due to global ischemic injury and that Akt activation and Bad phosphorylation by estradiol mediated these protective effects.

KEY WORDS: Akt, Bad, Estradiol, neuroprotection.

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The major gonadal sex hormone, estradiol, plays potent neurotrophic and neuroprotective roles in immature and adult brains [8, 11, 16]. Also, estradiol inhibits cell death as a response to a variety of neuronal stimuli, including excitotoxic amino acids and oxidative stress [3, 4]. In particular, clinical studies have shown that estradiol decreases the risk or severity of neurodegenerative conditions such as Alzheimer's disease, stroke, and Parkinson's disease [5, 10, 12]. The physiological level of estradiol prevents ischemia-induced hippocampal injury in gerbils through the inhibition of apoptotic signaling cascades [6]. Also, numerous studies have demonstrated that estradiol inhibits cell death caused by ischemic brain injury, by decreasing the extent of apoptotic cell death and enhancing cell survival signals [15, 16].

The phosphatidylinositol 3-kinase (PI3-K)/Akt signal pathway plays an important role to regulate several cellular function including cell growth, apoptosis, and survival [2]. In the presence of survival factors, activated Akt phosphorylates serine residues of Bad and attenuates its action. The phosphorylated Bad interacts with 14-3-3, which prevents Bad from binding with Bcl-x(L) at the mitochondrial membrane [19, 20]. 14-3-3 acts as an anti-apoptotic factor through interaction with pro-apoptotic molecules such as Bad [9]. Apoptotic stimuli induced the dephosphorylation of Bad, releasing it from 14-3-3 [19, 20]. Consequently, dephosphorylated Bad interacts with the anti-apoptotic protein Bcl-x(L), blocks Bcl-x(L)-dependent cell survival pathway, and induces apoptosis [19, 20]. Therefore, the phosphorylation of Bad is critical for the suppression of

apoptosis.

Estradiol was reported to induce the phosphorylation of Akt, noting that the PI3-K/Akt survival pathway mediates the neuroprotective effects of estradiol against brain injury [16]. Although several studies have demonstrated the neuroprotective effect of estradiol, little data is available regarding the activation of Bad. Therefore, in order to ascertain the neuroprotective effect of estradiol and elucidate the mechanism of estradiol action in global ischemia, the present study used a gerbil model of global ischemia.

MATERIALS AND METHODS

Animals, global ischemia and tissue collection: Female Mongolian gerbils (65–75 g) were obtained from the Experimental Animal Center, Gyeongsang National University (Jinju, South Korea) and were randomly divided into estradiol-treated group and non-treated group. Animals were maintained under controlled temperature (25°C) and lighting (14/10 light/dark cycle), and were allowed to have free access to food and water. To eliminate endogenous estradiol production, gerbils were bilaterally ovariectomized using a dorsal approach. And then gerbils were implanted with a time-releasing pellet containing 17 β -estradiol (0.36 mg, Innovative Research of America, Sarasota, FL, U.S.A.) or were not treated with estradiol. The estradiol pellet size was based upon findings of Jover *et al.* [6]. Two weeks after ovariectomy and treatment, animals were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg). A midline ventral incision was made in the neck, and both common carotid arteries were isolated, and occluded using non-traumatic aneurysm clips. Complete interruption of blood flow

* CORRESPONDENCE TO: KOH, P.O., Department of Anatomy, College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, South Korea.

was confirmed by observing the central artery in retina using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. Restoration of blood flow was directly observed under the ophthalmoscope. Sham-operated animals served as controls. On 1, 3, 5 day after the surgery, non-treated and 17 β -estradiol-treated animals (n=10 per group) were sacrificed. For the morphological study, animals were perfused transcardially with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). The brain were removed from the skull and fixed in the same fixative for 24–48 hr. Thereafter, the brains were embedded in paraffin, cut into 4 μ m thick sections, and stained with 1% cresyl violet.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL): TUNEL histochemistry was performed using the DNA Fragmentation Detection Kit (Oncogene Research Products, Cambridge, MA, U.S.A.). Briefly, the paraffin sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed PBS. The sections were treated 0.05% proteinase K for 5 min and blocked with 0.3% hydrogen peroxide in methyl alcohol for 10 min. After washes in PBS, sections were incubated with equilibration buffer followed by TdT enzyme in a humidified chamber at 37°C for 1 hr, and then a stop/wash buffer was applied for 30 min at 37°C. Finally, sections were incubated with peroxidase labeled anti-digoxigenin antibody for 30 min at room temperature. Positive cells were visualized using a diaminobenzidine substrate (Sigma chemical Co., St. Louis, MO, U.S.A.) and counterstained with 1% cresyl violet.

Western blot analysis: Animals were decapitated and brains were removed on 1, 3, 5 day after ischemic injury. The hippocampus regions were dissected. Tissues samples were snap frozen and lysed in buffer [1% Triton X-100, 1 mM EDTA in 1 $\times$ PBS (pH 7.4)] containing 10 μ M leupeptin and 200 μ M phenylmethylsulfonyl fluoride. The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's protocol. Total protein (30 μ g) was applied to each lane on to 10% SDS-polyacrylamide gels. After electrophoresis and immunoblotting, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the following antibodies: anti-Akt, anti-phosphoAkt⁴⁷³, anti-Bad, anti-phosphoBad (diluted 1:1000, Cell Signaling Technology, Beverly, MA, U.S.A.) as primary antibody. And the membrane was incubated with secondary antibody (1:5000, Pierce, Rockford, IL, U.S.A.) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) according to the manufacturer's protocol was used for detection.

Data analysis: All data are expressed as mean $\pm$ S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Student's *t* test. The difference for comparison was considered significant at **p*<0.05.

RESULTS

We observed the neuroprotective effect of estradiol in hippocampal CA1 region. On 1 day after ischemic injury, the hippocampal CA1 neuronal cell did not reveal any detectable cell death in both non-treated and estradiol-treated animals (Fig. 1A, 1B). However, some cells exhibited pyknotic nuclei in non-treated animals on 3 day after ischemic injury (Fig. 1C). On 5 day after injury, almost the cytosolic membrane of neuron were ruptured and irreversibly damaged in non-treated animals (Fig. 1E). However, in estradiol-treated animals, only a few neurons exhibited detectable neuronal death on 3 and 5 day after ischemia (Fig. 1D, 1F). We detected DNA fragmentation to clarify the extent of damage. The TUNEL staining was highly expressed within the cell nuclei of hippocampal CA1 pyramidal neurons in non-treated animals on 5 day after injury (Fig. 2A). However, positive cells of TUNEL staining did not observe in the estradiol-treated animals (Fig. 2B).

We investigated the activation of Akt and Bad by phosphorylation of Akt at Ser⁴⁷³ and Bad at Ser¹³⁶ in the hippocampal region after ischemic injury using western blot analysis (Figs. 3, 4). The level of pAkt was significantly decreased in non-treated animals on 1–5 day after ischemic injury. However, the levels of pAkt were maintained in estradiol-treated animals as the levels of controls. On 5 day

Fig. 1. Representative microphotographs of the ischemic hippocampal CA1 region on 1 (A and B), 3 (C and D), 5 (E and F) days after ischemic injury in non-treated gerbils (A, C, and E) and estradiol-treated gerbils (B, D, and F). Cresyl-violet staining was performed. Arrows indicate the pyknotic nuclei. Scale bar = 25 μ m.

Fig. 2. Representative microphotographs of TUNEL staining in hippocampal CA1 region on 5 day after ischemic injury in non-treated gerbils (A) and estradiol-treated gerbils (B). Arrows indicate positive cells. Scale bar = 25 μ m.

Fig. 3. Western blot analysis of pAkt in the hippocampus on 1, 3, 5 day after ischemic injury in non-treated gerbils and estradiol-treated gerbils. Control indicates sham-operated gerbils. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α -tubulin. Data ($n=5$) are represented as mean $\pm$ S.E.M. * $P<0.05$.

after injury, the level of pAkt was 0.69 ± 0.09 and 1.09 ± 0.11 in non-treated animals and estradiol-treated animals, respectively (Fig. 3). Also, the level of pBad was significantly decreased in non-treated animals on 1–5 day after ischemic injury. On 5 day after injury, the level of pBad was 0.63 ± 0.12 in non-treated animals, whereas it was 1.13 ± 0.17 in estradiol-treated animals (Fig. 4). Estradiol prevented injury-induced decline of pBad. The expression levels of Akt and Bad were consistently maintained in both

Fig. 4. Western blot analysis of pBad in the hippocampus on 1, 3, 5 day after ischemic injury in non-treated animals and estradiol-treated animals. Control indicates sham-operated gerbils. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α -tubulin. Data ($n=5$) are represented as mean $\pm$ S.E.M. * $P<0.05$.

non-treated animals and estradiol-treated animals.

DISCUSSION

It was known that transient global ischemia leads to selective and delayed neuronal death, particularly of pyramidal neurons in the hippocampal CA1 [6]. Previous studies have demonstrated that estrogen prevents the loss of CA1 hippocampal neurons in gerbils after ischemic injury [13]. Also, Jover *et al.* [6] demonstrated that estrogen protects neuron against global ischemia-induced neuronal death and prevents activation of apoptotic signaling cascades in hippocampal CA1 region. In this study, gerbil model of ischemia was used to investigate the neuroprotective effect of estradiol against delayed and selective neuronal cell death. Our data showed that estradiol treatment significantly decreased the apoptotic cell death in hippocampal CA1 pyramidal neuron using TUNEL method, a marker for apoptotic cell death. Also, previous our studies showed the neuroprotective effect of estradiol in focal cerebral ischemic injury [17, 18]. Thus, we have clearly confirmed that estradiol mediates the neuroprotective effect through the inhibition of cell death in animal models for global and focal ischemia.

The PI3-K/Akt signal pathway is important for promoting cell survival and suppressing cell death. Singh *et al.* [14]

demonstrated that estrogen activates the PI3-K pathway to inhibit apoptosis and support neuronal survival. Moreover, it was demonstrated that the neuroprotective effects of estradiol against brain injury were mediated through the phosphorylation of Akt [16]. Although previous studies have demonstrated the neuroprotective effect of estradiol through activation of the PI3-K/Akt pathway, there is little information regarding the activation of Akt downstream including target such as Bad. This study focused on the activation of Akt and phosphorylation of Bad through the use of estradiol in ischemic brain injury. Ischemia-induced neuronal death was not detected on 1 day after induction of injury. However, our data showed that the level of pAkt decreased on 1 day after brain injury, and estradiol maintained the level of pAkt as the level of control. It is well known that activation of Akt contributes to the suppression of apoptosis [1]. Thus, our results suggest that the neuroprotective effect of estradiol against ischemic injury would be attributed to the long-lasting activation of the Akt.

In the presence of survival factor, Akt is known to phosphorylate Bad at the Ser¹³⁶ residue that is critical for sequestration to 14-3-3 [7]. However, apoptotic stimuli induce the dephosphorylation of Bad. Dephosphorylated Bad interacts with Bcl-x(L), blocks Bcl-x(L)-dependent cell survival pathway, and promotes cell death [19, 20]. Our data showed that the level of pBad decreased after brain injury in the absence of estradiol, and estradiol prevented down-regulation of pBad levels. Actually, brain injury decreased the levels of pBad and estradiol maintained the levels of pBad as the levels of control. The decrease of pBad levels induces neuronal cell death. However, estradiol prevents down-regulation of pBad levels from global ischemic injury, thereby protecting against brain injury. In previous our studies, we demonstrated that estradiol prevents the focal ischemic injury-induced decrease of Akt activation and Bad phosphorylation in middle cerebral artery occlusion [17, 18]. Furthermore, this study showed the effect of estradiol in global ischemia model. Thus, we demonstrate that estradiol prevents cell death caused by ischemic brain injury through the activation of Akt and its downstream target such as Bad. In conclusion, we suggest the fact that estradiol plays a neuroprotective role by preventing the injury-induced decline of pAkt and pBad.

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