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Estradiol prevents the injury-induced decrease of 90 ribosomal S6 kinase (p90RSK) and Bad phosphorylation

Phil Ok Koh *

Department of Anatomy, College of Veterinary Medicine and Research Institute of Life Science, Gyeongsang National University, 900 Gajwa-dong, Jinju 660-701, South Korea

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Abstract

Estradiol prevents neuronal cell death through the activation of cell survival signals and the inhibition of apoptotic signals. This study investigated whether estradiol modulates the anti-apoptotic signal through the activation of Raf-MEK-ERK and its downstream targets, including 90 ribosomal S6 kinase (p90RSK) and Bad. Adult female rats were ovariectomied and treated with estradiol prior to middle cerebral artery occlusion (MCAO). Brains were collected 24 h after MCAO and infarct volumes were analyzed. We confirmed that estradiol significantly reduces infarct volume and decreases the positive cells of TUNEL staining in the cerebral cortex. Estradiol prevents the injury-induced decrease of Raf-1, MEK1/2, and ERK1/2 phosphorylation. Also, it inhibits the injury-induced decrease of p90RSK and Bad phosphorylation. Further, in the presence of estradiol, the interaction of phospho-Bad and 14-3-3 increased, compared with that of oil-treated animals. Our findings suggest that estradiol prevents cell death due to brain injury and that Raf-MEK-ERK cascade activation and its downstream targets, p90RSK, Bad phosphorylation by estradiol mediated these protective effects.
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Keywords: Estradiol; Neuroprotection; p90RSK; Bad

Estradiol is the major female sex hormone and serves many important functions. In addition to its classic role in reproduction, it also plays potent neurotrophic and neuroprotective roles in immature and adult brains [8]. Estradiol prevents cell death as a response to a variety of neuronal stimuli, including excitotoxic amino acids, β-amyloid toxicity, and oxidative stress [2,5]. In particular, clinical studies have shown that estradiol decreased the risk or severity of neurodegenerative conditions such as Alzheimer's disease [6,11], stroke, and Parkinson's disease [13]. Also, estradiol has been known to inhibit cell death caused by ischemic brain injury, by decreasing the extent of apoptotic cell death and enhancing cell survival signals [18].

The mitogen-activated protein kinase (MAPK, ERK1/2) signaling pathway regulates a variety of cellular processes that include cell proliferation, differentiation, survival, and apoptosis [12]. Raf and MAPK kinase (MEK) are the upstream enzymes in the MAPK pathway. It is known that a variety of extracellular signals activate MEK to phosphorylate ERK1/2 [12]. Also, ERK1/2 can phosphorylate and activate the 90 kDa ribosomal S6 kinase (p90RSK), which then leads to the phosphorylation and inactivation of the pro-apoptotic bcl-2 family, Bad [1,4,14]. The phosphorylated Bad interacts with 14-3-3, which prevents Bad from binding with Bcl-x(L) at the mitochondrial membrane [20,21]. 14-3-3 acts as an anti-apoptotic factor through interaction with pro-apoptotic molecules such as Bad [10]. Consequently, the Raf-MEK-ERK signal cascade prevents apoptosis through the phosphorylation of downstream targets, p90RSK and Bad.

Previous studies have shown that the two isoforms of MAPK, ERK1/2, were activated by phosphorylation in response to estrogen, leading to attenuation of neuronal injury during glutamate- and β-amyloid peptide-induced toxicity [9,16]. Also, we previously reported that estradiol plays a neuroprotective role by preventing the injury-induced decrease of Akt activation and its downstream target, Bad phosphorylation [19]. Although several studies have demonstrated the neuroprotective effect of estradiol, little data is available regarding the activation of ERK1/2 downstream targets. Thus, this study examined the neuroprotective effect of estradiol against stroke-like ischemic injury and investigated the role of estradiol in the mediation

* Tel.: +82 55 751 5809; fax: +82 55 751 5803.
E-mail address: pokoh@gsnu.ac.kr.

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of anti-apoptotic signaling through the activation of Raf-MEK-ERK and its downstream target, including p90RSK, Bad, and 14-3-3.

Female Sprague–Dawley rats (225–250 gm, 12 weeks age, $n = 60$) were purchased from Samtako Co. (Animal Breeding Center, Korea) and were randomly divided into two groups, oil-treated group and estradiol-treated group ($n = 30$ per 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, rats were bilaterally ovariectomized using a dorsal approach. And then implanted with a silastic capsule containing Sesame oil (Sigma, St. Louis, MO) or 17β-estradiol (180 μg/ml, Sigma, St. Louis, MO). This paradigm of estradiol treatment produces levels of estradiol equivalent to basal circulating levels observed during the rat estrous cycles [2]. Two weeks after ovariectomy and treatment, rats underwent middle cerebral artery occlusion (MCAO) to induce cerebral ischemia.

Before surgery, animals were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg). MCAO was carried out as described previously [7]. Briefly, the right common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 4/0 monofilament nylon suture with its tip slightly rounded by heat was inserted through the internal carotid artery to the base of the middle cerebral artery, thus occluding blood flow to the cortex and striatum. At 24 h after the onset of permanent occlusion, animals were decapitated and the brains were removed. These brains were cut into 2 mm thick coronal slices at the optic chiasm level, the coronal levels at which the largest ischemic infarct is observed [15].

These slices were incubated for 20 min in a 2% triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MA, USA) and fixed in 10% formalin. The stained slices were photographed by a Nikon CoolPIX990 digital camera and measured for the ischemic lesion by Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The largest ischemic lesion in brain region was measured, respectively ($n = 15$ per group). The ischemic lesion percentage of each slice was calculated by the ratio of the infarction area to the whole slice area. After TTC staining and lesion area analysis, the slices were embedded with paraffin and sectioned for TUNEL staining. TUNEL histochemistry was performed using the DNA Fragmentation Detection Kit (Oncogene Research Products, Cambrige, MA, USA). In the TUNEL stained sections, one field for each section was selected from cerebral cortex. TUNEL-positive cells were quantified using light microscopy at magnification (×40). The total cell number and TUNEL-positive cell number were obtained in each field. The percentage of TUNEL-positive cells is described as the percentage of the number of TUNEL-positive cells to the total number of cells in each field.

For Western blot and immunoprecipitation analyses, the slices were dissected into ipsilateral and contralateral cerebral cortex ($n = 15$ per group). Tissues samples were snap frozen and lysed in buffer (1% Triton X-100, 1 mM EDTA in 1× PBS (pH 7.4)) containing 10 μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. 30 μg of total protein was applied to each lane on to 10% SDS-polyacrylamide gels. After electrophoresis and immunoblotting, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the following antibodies: anti-Raf-1, anti-phospho-Raf-1(Ser338), anti-MEK1/2, anti-phospho-MEK1/2(Ser217/Ser221), anti-ERK1/2, anti-phospho-ERK1/2(Thr202/Thr204), anti-p90-RSK, anti-phospho-p90RSK(Ser383), anti-Bad, anti-phospho-Bad(Ser112), (diluted 1:1000, Cell Signaling Technology, Beverly, MA, USA), and 14-3-3β antibody (diluted 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibody. And the membrane was incubated with secondary antibody (1:5000, Pierce, Rockford, IL, USA) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's protocol was used for detection.

For the immunoprecipitation of 14-3-3, 200 μg of total protein was used and was pre-cleared with Protein-A agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to remove nonspecific-binding proteins. The precleared samples were

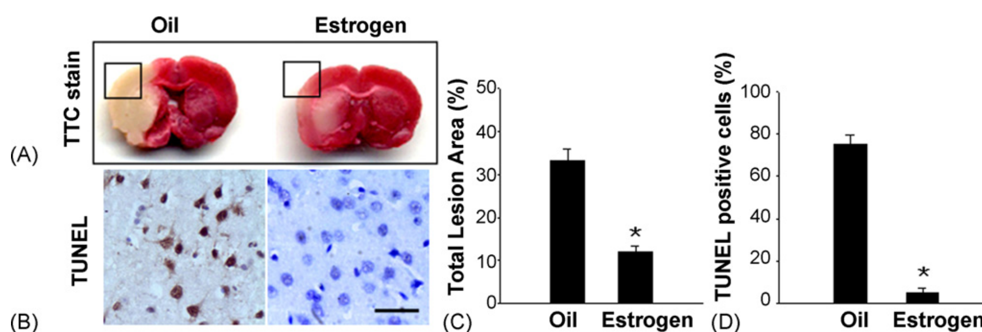

Fig. 1. Representative photos of TTC stain and TUNEL histochemistry in the cortex from oil- and estradiol-treated rats prior to MCAO. (A) Brain sections were stained by TTC. The ischemic area remained white, while the intact area was stained red. Estradiol treatment significantly protected the cerebral cortex from ischemic brain injury. (B) Photographs of TUNEL staining in the cerebral cortex from an oil- and an estradiol-treated rat. These photos indicate the square areas of A. Positive cells of TUNEL staining were markedly decreased in estradiol-treated rats. Scale bar = 100 μm. (C) The percentage of ischemic lesion area was calculated as lesion area/whole brain section area. (D) The percentage of TUNEL-positive cells was described as the number of TUNEL-positive cells/total number of cells in each field. Data ($n = 15$) are represented as mean ± S.E.M.; $^*P < 0.05$.

incubated with anti-14-3-3β antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for overnight at 4 °C. And then the Immune complex was then precipitated with protein A/G agarose beads for 2 h at 4 °C. The protein-bead complex was then washed and collected by centrifugation. The samples were processed as described for western blot analysis. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, USA) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, USA). The results are the mean of five independent experiments.

All data are expressed as mean ± 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 $P < 0.05$.

Estradiol administration significantly reduced the infarct volume by over 60%, compared with oil-treated control (Fig. 1A and C). In particular, the protective effect of estradiol appears significantly in the cerebral cortex. However, estradiol did not protect against injury in the striatum. The ischemic lesion area was 34.52% and 12.35% in oil-treated and estradiol-treated animals, respectively (Fig. 1C). In oil-treated animals, the number of TUNEL positive cells significantly increased in the infarct region, whereas it was markedly reduced in the cerebral cortex of estradiol-treated animals (Fig. 1B and D). 72.5 ± 2.5% and 4.9 ± 1.5% of cells were TUNEL positive in the ipsilateral cortex of oil- and estradiol-treated animals, respectively.

We investigated the activation of Raf-1 and MEK1/2 by phosphorylation of Raf-1 at Ser^{338} and MEK1/2 at $Ser^{217/221}$ in the cerebral cortex (Fig. 2). Western blot showed that brain injury induced decrease in phospho-Raf-1 and phospho-MEK1/2 levels, and estradiol prevented injury-induced down regulation of phospho-Raf-1 and phospho-MEK1/2. The level of phospho-Raf-1 was 0.73 ± 0.08 and 0.86 ± 0.15 in the ipsilateral cortex of oil- and estradiol-treated animals, respectively (Fig. 2A). The level of phospho-MEK1/2 was 0.65 ± 0.05 and 1.07 ± 0.09 in the ipsilateral cortex of oil- and estradiol-treated animals, respectively (Fig. 2B). In the presence of estradiol, the level of phospho-ERK1/2 was maintained in the ipsilateral cortex as the level of contralateral cortex. The level of phospho-ERK1/2 was 0.96 ± 0.15 in the ipsilateral cortex of estradiol-treated animals. Whereas it was 0.78 ± 0.08 in oil-treated animals (Fig. 2C). The level of phospho-p90RSK was 0.72 ± 0.07 and 1.04 ± 0.09 in

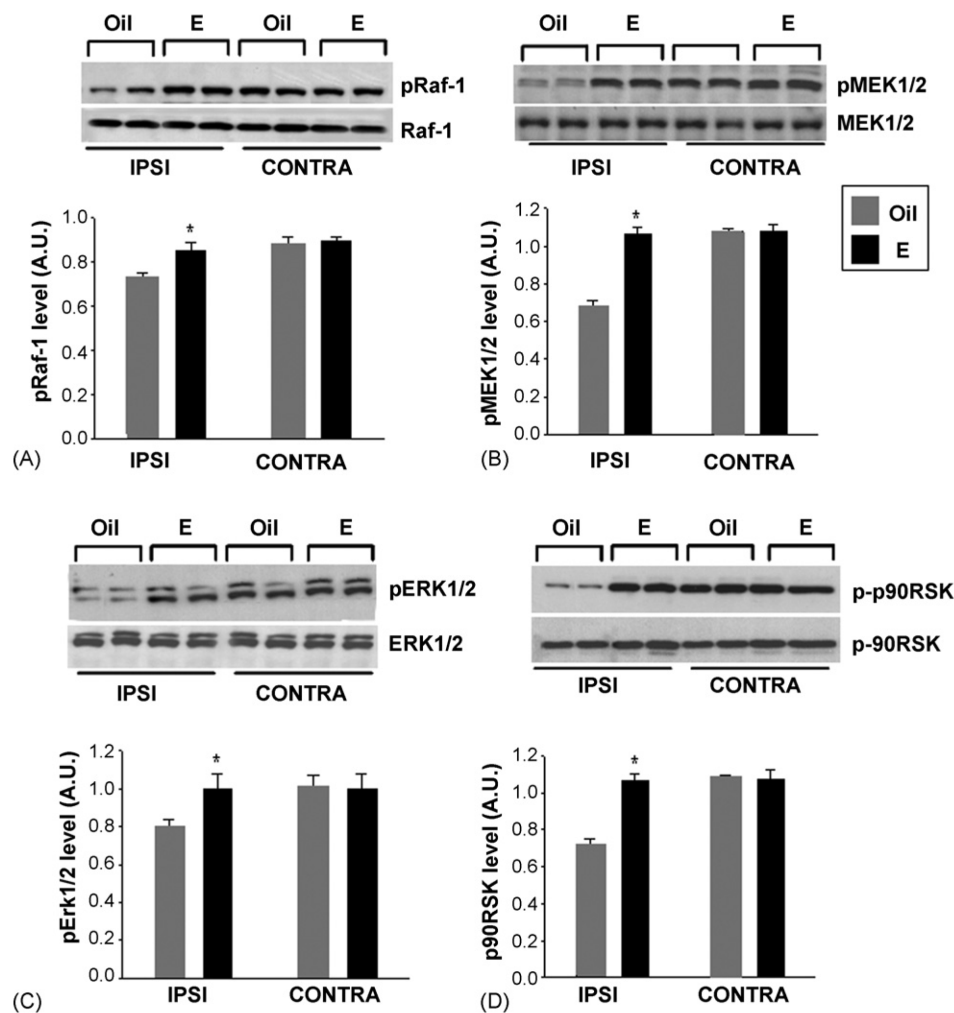

Fig. 2. Western blot analysis of phospho-Raf-1 (A), phospho-MEK1/2 (B), phospho-ERK1/2 (C), and phospho-p90RSK (D) in the ipsilateral and contralateral cortex. Rats were treated oil (labeled Oil) or estradiol (labeled E) prior to MCAO. Each lane represents an individual experimental animal. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α-tubulin. Data ($n = 15$) are represented as mean ± S.E.M.; $^{*}P < 0.05$.

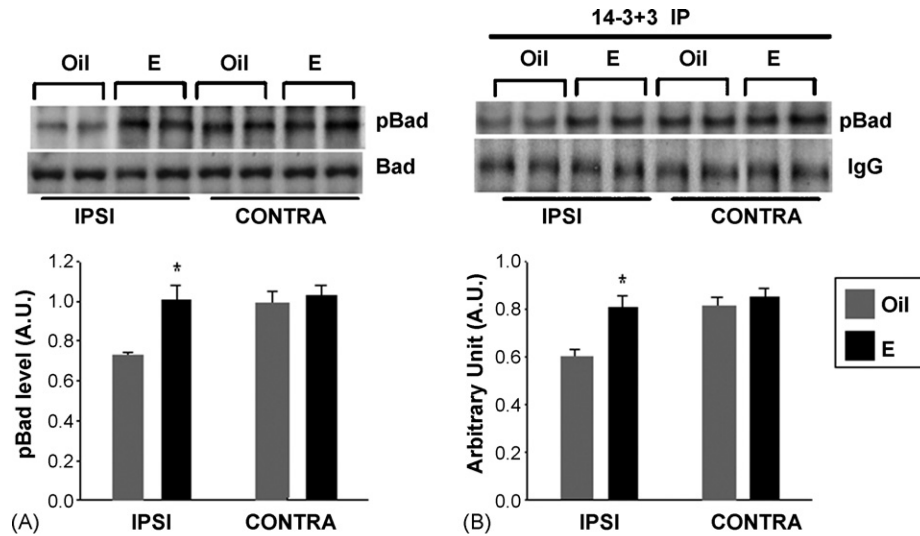

Fig. 3. Western blot analysis of phospho-Bad (A) and immunoprecipitation analysis of phospho-Bad and 14-3-3 interaction (B) in the ipsilateral and contralateral cortex. Rats were treated with oil (labeled Oil) or estradiol (labeled E) prior to MCAO. Each lane represents an individual experimental animal. Densitometric analysis of phospho-Bad and 14-3-3 interaction in the ipsilateral cortex is represented as an arbitrary unit (A.U.) that was normalized by IgG. Data ($n = 15$) are represented as mean ± S.E.M.; $^{*}P < 0.05$.

the ipsilateral cortex of oil- and estradiol-treated animals, respectively (Fig. 2D). Also, the level of phospho-Bad was 0.73 ± 0.05 and 0.98 ± 0.11 in the ipsilateral cortex of oil- and estradiol-treated animals, respectively (Fig. 3A). Fig. 3B showed the interaction of phospho-Bad and 14-3-3 by immunoprecipitation in both oil- and estradiol- treated animals. The interaction of phospho-Bad and 14-3-3 decreased in the ipsilateral cortex of oil-treated animals. The binding levels of 14-3-3/phospho-Bad were 0.59 ± 0.07 and 0.80 ± 0.09 in the ipsilateral cortex of oil-and estradiol-treated animals, respectively (Fig. 3B).

Previous studies have demonstrated that the physiological levels of 17β-estradiol protect the brain against ischemic injury by MCAO and it exerts potent neuroprotective actions in cultured explants of the cerebral cortex [15]. Also, Wilson et al. [18] demonstrated that estrogen significantly diminished the number of apoptotic nuclei following injury. In this study, our data showed that estradiol treatment significantly reduced the infarct volume in the cerebral cortex. However, estradiol did not prevent cell death in the striatum region. The previous studies reported that region-specific effects partially reflect differential blood perfusion to the cortex and striatum following MCAO: MCAO blocks blood flow to a greater extent in the striatum compared with the cortex [17]. Our results also showed that estradiol markedly decreased the number of apoptotic cells in the cerebral cortex using TUNEL method. Thus, we have clearly confirmed that estradiol mediates the neuroprotective effect through the inhibition of cell death, apoptosis.

The MAPK signaling cascade plays critical roles in the promoting cell survival and suppressing cell death [1,3,14]. Upon growth factor stimulation, a protein kinase cascade is initiated that sequentally activates Raf, MEK, ERK, and p90RSK. It was demonstrated that the neuroprotective effects of estradiol against oxidative stress and glutamte-induced toxicity were mediated through the phosphorylation of MEK and ERK1/2 [9,16]. Although previous studies have demonstrated the neuroprotective effect of estradiol through activation of the MEK-ERK1/2, there is little information regarding the activation of ERK1/2 downstream targets, such as p90RSK and Bad. This study focused on the activation of p90RSK and Bad as well as on the interaction of Bad and 14-3-3 through the use of estradiol in ischemic brain injury. Our data showed that the levels of phospho-Raf-1, phospho-MEK1/2, phospho-ERK1/2, and phospho-p90RSK decrease in cases of brain injury, and estradiol prevents down-regulation of these genes levels, thereby protecting against brain injury.

Raf-MEK-ERK pathway can prevent apoptosis through the phosphorylation of protein that regulate apoptosis. Actually, p90RSK, a substrate of ERK1/2, is known to phosphorylate Bad at the Ser112 residue, which contributes to prevent apoptosis [21]. The phosphorylated Bad is bound and sequestered in the cytosol by the chaperone protein 14-3-3. However, dephosphorylated Bad promotes apoptosis by binding to Bcl-x(L) and releases proapoptotic Bax from the interaction of Bcl-x(L) and Bax. Sequentially, Bax promotes the release of cytochrome *C* and the activation of caspase cascade, initiates apoptosis. Thus, the phosphorylation of Bad is critical for the inhibition of apoptosis. In the absence of estradiol, the levels of phospho-p90RSK and phospho-Bad significantly decreased in the injury region. Estradiol prevents the injury-induced declines of phospho-p90RSK and phospho-Bad. The phosphorylated Bad interacted with the 14-3-3. These interactions prevented Bad from binding with Bcl-x(L) at the mitochodrial membrane. Therefore, the interaction of Bad and 14-3-3 is necessary for suppression of cell death. Our results demonstrated that estradiol prevents the injury-induced decrease of p90RSK phosphorylation and Bad phosphorylation. As a next step, estradiol maintains the interaction of phospho-Bad and 14-3-3 as the levels of intact region. Consequently, estradiol prevents the injury-induced a decline of the binding of phospho-Bad and 14-3-3. The interaction of phospho-Bad and 14-3-3 blocks the action of Bax,

pro-apoptotic factor. Thus, we demonstrate that estradiol prevents cell death caused by ischemic brain injury. In conclusion, we suggest that estradiol plays a neuroprotective role by preventing the injury-induced decline of ERK1/2 downstream targets, phospho-p90RSK and phospho-Bad.

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