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ScienceDirect
Neuroscience Letters 412 (2007) 68–72
Neuroscience Letters
www.elsevier.com/locate/neulet

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

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Received 18 September 2006; received in revised form 14 October 2006; accepted 17 October 2006

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.

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

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doi:10.1016/j.neulet.2006.10.060

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

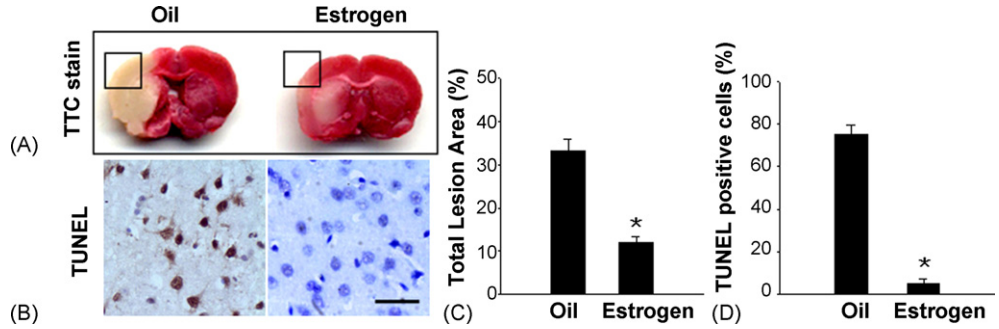

Fig. 1. Representative photos of TTC stain and TUNEL histochemistry in the cortex from oil- and estradiol-treated rats prior to MCAO. (A) Brain sections were stained by TTC. The ischemic area remained white, while the intact area was stained red. Estradiol treatment significantly protected the cerebral 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.; $^{*}P < 0.05$.