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Putative membrane-bound estrogen receptors possibly stimulate mitogen-activated protein kinase in the rat hippocampus

Yasutomi Kuroki, Kouji Fukushima, Yasunari Kanda, Katsushige Mizuno, Yasuhiro Watanabe *

Department of Pharmacology, National Defense Medical College, 3-2 Namiki, Tokorozawa 359-8513, Japan

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Abstract

We investigated whether 17β-estradiol affects the activity of extracellular signal-regulated kinase (ERK) in the central nervous system in vivo. 17β-Estradiol was administered intracerebroventricularly, and then ERK activity in the rat hippocampus was measured. We found that ERK activity in the rat hippocampus had increased to approximately threefold its basal level at 5 min. This rapid effect was mimicked by the membrane-impermeable estradiol, bovine serum albumin-conjugated 17β-estradiol, and was not inhibited by tamoxifen and 7α,9-(4,4,5,5,5-pentafluoropentylsulphinyl)nonylestra-1,3,5(10)-triene-3,17β-diol (ICI 182780), classical nuclear estrogen receptor antagonists. These data suggest that the rapid activation of ERK by estradiol in vivo is mediated through a putative membrane estrogen receptor in the rat hippocampus. © 2000 Published by Elsevier Science B.V.

Keywords: Estrogen; Non-genomic effect; ERK (extracellular signal-regulated kinase); Hippocampus, rat

1. Introduction

Ovarian steroids are of prime importance in the normal maintenance of brain function. The loss of these steroids at menopause may account, in part, for the cognitive decline and neurodegeneration associated with neurodegenerative diseases, such as Alzheimer's disease (Simpkins et al., 1994). In fact, estrogen treatment has been shown to enhance performance on some tests of memory in women with Alzheimer's disease (Ohkura et al., 1994). In addition, estrogens have been shown to protect neurons in male rats subsequent to moderate traumatic brain injury (Emerson et al., 1993), and to enhance neuronal survival resulting from oxidative stress, excitotoxicity, and β-amyloid (Behl et al., 1995; Goodman et al. 1996; Green et al., 1996); high doses of estrogen have also been shown to protect neurons from ischemia (Chen et al., 1998). However, the mechanisms of these neuroprotective effects of estrogens have not been elucidated.

Estrogen causes most of its effects by means of direct binding to specific nuclear receptors, which then act as transcriptional activators. However, recent studies have indicated the presence of non-genomic effects of estrogen. For example, estrogen plays an important role in ErbB2-mediated signaling, as a member of the EGF receptor family (Matsuda et al., 1993). Estrogen potentiates kainate currents in the hippocampus via cAMP-dependent phosphorylation (Gu and Moss, 1996), increases cAMP accumulation in breast cells (Aronica et al., 1994), and rapidly activates mitogen-activated protein kinase (MAPK) in neuroblastoma cells (Watters et al., 1997) and in non-neuronal cells (Migliaccio et al., 1996; Endoh et al., 1997). Thus, evidence has accumulated to support the hypothesis that cross communication occurs between the actions of estrogen and growth factors.

Extracellular signal-regulated kinase (ERK), one of the most important MAPKs, is located in the common downstream of many growth factor signal cascades. In the experiments reported herein, in order to investigate the effects of 17β-estradiol on the ERK cascade in vivo, we explored whether or not 17β-estradiol affects the activity

* Corresponding author. Tel.: +81-42-995-1484; fax: +81-42-996-5191.

E-mail address: ndmcphar@sa2.so-net.ne.jp (Y. Watanabe).

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of ERK in the rat hippocampus through putative membrane-bound estrogen receptors.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (8–9 weeks old, 250–330 g) were individually housed in a temperature-controlled environment on a 12:12 h light/dark cycle with the lights on at 07.00–19.00 h and with ad lib access to food and water.

2.2. Reagents

17β-Estradiol, bovine serum albumin-conjugated 17β-estradiol, 17α-estradiol and tamoxifen were obtained from Sigma. 7α,9-(4,4,5,5,5-Pentafluoropentylsulphinyl)nonylestra-1,3,5(10)-triene-3,17β-diol (ICI 182780) was a gift from Zeneca Pharmaceuticals (Cheshire, UK).

2.3. Stereotaxic injection of drugs

Rats were anesthetized with 50 mg/kg sodium pentobarbital prior to stereotaxic surgery. The head was placed in a stereotaxic apparatus with the skull exposed. A small burr hole was drilled in the left hemisphere for purposes of drug administration and two additional holes were drilled bilaterally for purposes of freezing. A stainless steel needle (26-gauge) was inserted into the left ventricle (coordinates: 0.9 mm posterior from the bregma, 2.0 mm from the midline, and 3.5 mm from the brain surface) and connected to a Hamilton syringe. Five microliters of a solution containing the drug being investigated (e.g., 17β-estradiol) was constantly infused at a rate of 5 μl/min. The needle was left in place for 4 additional min to allow diffusion of the drug solution. At the appropriate time after the injection, the brain was frozen via the freezing holes with liquid nitrogen for 3 min. Thereafter, the rat was decapitated, and the head was frozen in liquid nitrogen for 10 min and stored at −80°C until freeze-drying.

2.4. Sample preparation

The heads were freeze-dried with an appropriate apparatus for 60 h. The CA regions and the dentate gyrus regions of the hippocampi were excised from the dried brains. These regions were sonicated briefly in an ice-cold homogenization buffer (50 mM 3-morpholinopropanesulfonic acid, 1 mM dithiothreitol, 10 mM KCl, 0.5 mM $MgCl_2$, 1 mM orthovanadate, 1 mM EDTA, 1 mM EGTA, 1 mM ouabain, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 mM β-glycerophosphate, and 40 μg/ml phenylmethanesulfonyl fluoride). Protein concentrations in the samples were determined by using a bicinchoninic acid protein assay reagent kit (Pierce), in accordance with the manufacturer's instructions. The samples were stored at −80°C until the kinase assay was performed.

2.5. MAPK (ERK) assay

ERK activity was measured using an EGF receptor peptide as a substrate with a MAPK enzyme assay system (Amersham), in accordance with the manufacturer's instructions. Briefly, the samples were incubated for 30 min at 30°C along with EGF receptor peptide as the substrate and [γ-^{32}P]ATP (3000 Ci/mmol; NEN Life Sciences Products), and scintillation counting.

2.6. MAPK (ERK) phosphorylation

An antibody, which recognizes the dual threonine and tyrosine phosphorylation sequence from MAPK (Phospho-p44/p42 MAPK Antibody; New England BioLabs), was used to evaluate ERK phosphorylation. Five micrograms of total protein from aliquots of the samples, obtained as described above, were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 8.3% polyacrylamide gels. After transfer to polyvinylidene difluoride membranes (Millipore), the membranes were blocked with 5% milk in 0.2% Tween 20 in phosphate-buffered saline (TPBS) and incubated with Phospho-p44/p42 MAPK antibody. Membranes were then incubated in horseradish peroxidase-conjugated secondary antibody (New England BioLabs) in TPBS, and the results were visualized by chemiluminescence (Amersham). To verify equal loading of protein across samples, the same membranes were then stripped of antibody in 62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol at 50°C. Samples were washed in TPBS, incubated with an antibody recognizing ERK (p44/p42 MAPK antibody; New England BioLabs), and examined by chemiluminescence.

3. Results

To investigate whether 17β-estradiol activates ERK, we performed time-course and dose–response studies. The ERK activity at both CA and dentate gyrus regions in the adult rat hippocampus increased approximately threefold from its basal level at 5 min after the administration of 10 μM 17β-estradiol, and decreased back to the basal level at 15 min (Fig. 1). Dose–response studies showed that 10 μM 17β-estradiol caused the full activation of ERK (Fig. 2), estradiol was used in this concentration in the subsequent experiments. These data indicate that estrogen induces the rapid and transient activation of ERK in the rat hippocampus in vivo.

To determine whether this rapid effect of estrogen occurs by any mechanism other than a nuclear estrogen

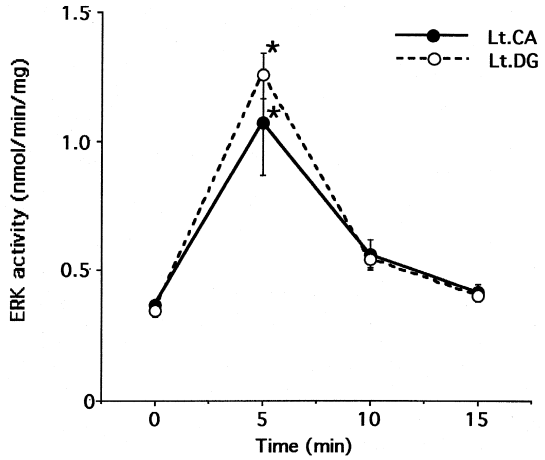

Fig. 1. Effect of 10 μM 17β-estradiol administration on ERK activity. 17β-Estradiol (10 μM) was administered to rats intracerebroventricularly. After the indicated time, the brains were frozen. Sample preparations and ERK assays were done as described in Section 2. Lt.CA, the CA region of the left hippocampus; Lt.DG, the dentate gyrus region of the left hippocampus. Values are means ± S.E.M.; $n = 4–6$. $^{*}P < 0.05$ as compared with time 0 (Mann–Whitney *U*-test).

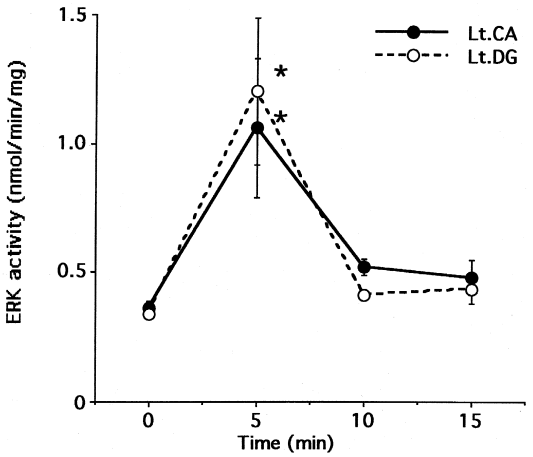

Fig. 3. Effect of the administration of 10 μM bovine serum albumin-conjugated 17β-estradiol on ERK activity. Bovine serum albumin-conjugated 17β-estradiol (10 μM) was administered to rats intracerebroventricularly. After the indicated time, the brains were frozen. Sample preparations and ERK assays were done as per described in Section 2. Lt.CA, the CA region of the left hippocampus; Lt.DG, the dentate gyrus region of the left hippocampus. Values are means ± S.E.M.; $n = 4–6$. $^{*}P < 0.05$ as compared with time 0 (Mann–Whitney *U*-test).

receptor, we used membrane-impermeable bovine serum albumin-conjugated 17β-estradiol. In a previous study, bovine serum albumin-conjugated 17β-estradiol did not activate estrogen response element-mediated transcription, due to its inability to enter the cell (Watters et al., 1997). Bovine serum albumin-conjugated 17β-estradiol did activate ERK; the maximal activity of ERK activated by bovine serum albumin-conjugated 17β-estradiol was similar to that activated by 17β-estradiol at 5 min after administration (Fig. 3). At 15 min, ERK activity decreased to the basal level. These data suggest that estrogen may have acted at the level of the neuronal membrane to initiate the ERK cascade under these in vivo experimental conditions.

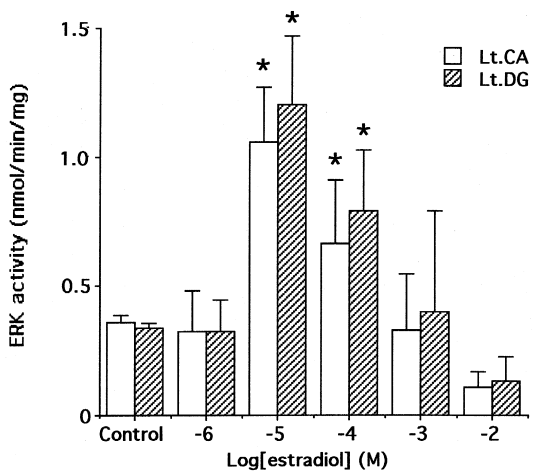

Fig. 2. Dose-dependent effects of 17β-estradiol in the rat hippocampus on ERK activity. Vehicle (control), or 17β-estradiol at indicated concentrations, were administered to rats intracerebroventricularly. After 5 min, the brains were frozen. Sample preparations and ERK assays were done as described in Section 2. Lt.CA, the CA region of the left hippocampus; Lt.DG, the dentate gyrus region of the left hippocampus. Values are means ± S.E.M.; $n = 4–6$. $^{*}P < 0.05$ as compared with control (Mann–Whitney *U*-test).

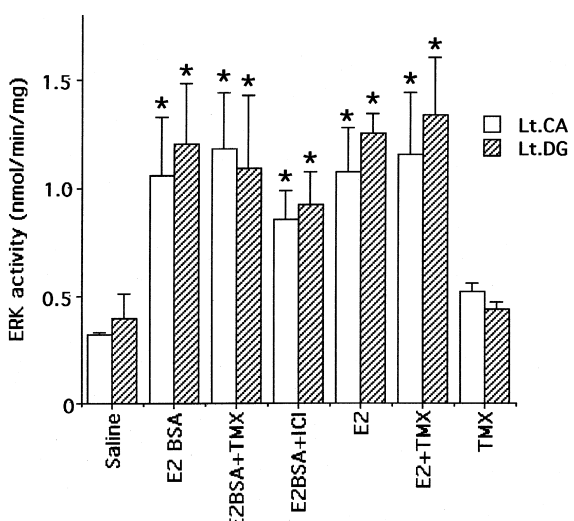

Fig. 4. Effects of the administration of tamoxifen or ICI 182780 on the activation of ERK by estrogen. Rats were intracerebroventricularly administered saline, 10 μM bovine serum albumin-conjugated 17β-estradiol alone (E2BSA), 10 μM bovine serum albumin-conjugated 17β-estradiol + 1 mM tamoxifen (E2BSA + TMX), 10 μM bovine serum albumin-conjugated 17β-estradiol + 1 mM ICI 182780 (E2BSA + ICI), 10 μM 17β-estradiol alone (E2), 10 μM 17β-estradiol + 1 mM tamoxifen (E2 + TMX) or 1 mM tamoxifen alone (TMX). After 5 min, the brains were frozen. Sample preparations and ERK assays were done as described in Section 2. Lt.CA, the CA region of the left hippocampus; Lt.DG, the dentate gyrus region of the left hippocampus. Values are means ± S.E.M.; $n = 4–6$. $^{*}P < 0.05$ as compared with saline (Mann–Whitney *U*-test).

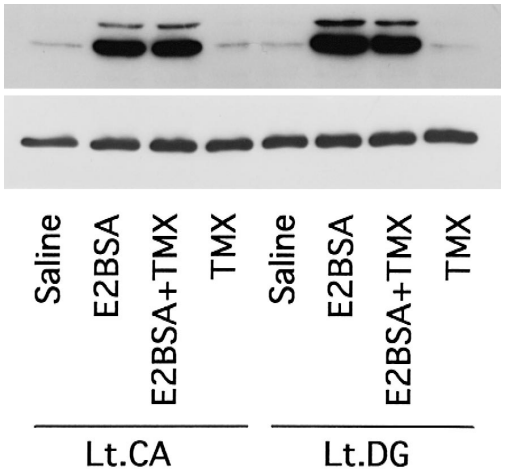

Fig. 5. Effects of the administration of tamoxifen on the phosphorylation of ERK by bovine serum albumin-conjugated 17β-estradiol. Rats were intracerebroventricularly administered saline, 10 μM bovine serum albumin-conjugated 17β-estradiol alone (E2BSA), 10 μM bovine serum albumin-conjugated 17β-estradiol + 1 mM tamoxifen (E2BSA + TMX), or 1 mM tamoxifen alone (TMX). After 5 min, the brains were frozen. Sample preparations and Western blotting were done as described in Section 2. Representative data of immunoblots are shown. The top panel represents phospho ERK. The bottom panel represents the same blots, which were stripped and reprobed for ERK protein as described in Section 2. Lt.CA, the CA region of the left hippocampus; Lt.DG, the dentate gyrus region of the left hippocampus.

Tamoxifen and ICI 182780 are nuclear estrogen receptor antagonists, which compete with estradiol for the binding of the nuclear estrogen receptor. To confirm that these rapid effects of estrogen do not occur via the classical nuclear estrogen receptor, we examined the effect of tamoxifen and ICI 182780 on the ERK activation by estrogen. The effects of both bovine serum albumin-conjugated 17β-estradiol and 17β-estradiol were not blocked by the simultaneous administration of 1 mM tamoxifen and 1 mM ICI 182780, although these agents alone did not affect the basal level of ERK activity (Fig. 4 and data not shown). In addition, the ERK activation corresponded with the phosphorylation of ERK (Fig. 5). These data suggest that the rapid activation of ERK by estradiol in vivo is mediated in a manner independent of the nuclear estrogen receptor in the hippocampus.

4. Discussion

In the present study, 17β-estradiol induced the rapid and transient activation of ERK in the rat hippocampus in vivo (Fig. 1). This rapid activation of ERK was mimicked by bovine serum albumin-conjugated 17β-estradiol, the membrane-impermeable estradiol (Fig. 3). Moreover, the effects of estrogen were not inhibited by nuclear estrogen receptor antagonists, tamoxifen and ICI 182780 (Fig. 4). Thus, we demonstrated that the rapid activation of ERK by estradiol in vivo is mediated through a putative membrane-bound estrogen receptor.

Evidence has accumulated to support the existence of non-genomic effects of estrogen. In the present study, estrogen caused the rapid and transient activation of ERK in the rat hippocampus in vivo (Figs. 1 and 3). These findings are consistent with those of previous in vitro studies (Watters et al., 1997; Migliaccio et al., 1996; Endoh et al., 1997). This rapid effect seems to be one of the non-genomic actions of estrogen, which occurs independent of estrogen response element-mediated transcription, because at least a few hours are required for estrogen to induce estrogen response element-mediated transcription.

Moreover, we demonstrated that the nuclear estrogen receptor antagonists, tamoxifen and ICI 182780, did not inhibit the effects of estrogen (Fig. 4). However, it has been considered controversial in previous studies that both of these estrogen receptor antagonists were unable to inhibit the rapid activation of ERK by estrogen; specifically, neither tamoxifen nor ICI 182780 exerted an influence on the effect of estrogen on ERK in a human neuroblastoma cell line (Watters et al., 1997), and in rat cerebral cortical explants (Singh et al., 1999). In contrast, the ICI 182780 compound blocked the effect of estrogen on ERK activation in a non-neuronal mammary tumor cell line, MCF-7 (Migliaccio et al., 1996). The variety of effects of estrogen antagonists may be due to cell specificity. Whereas the inhibitory effects of these estrogen receptor antagonists are associated with their ability to prevent the transcriptional activation of estrogen response element-containing genes, the effects of tamoxifen and ICI 182780 in this study may be associated with the non-genomic effects of estrogen through the activation of a membrane-bound estrogen receptor.

A previous report suggested that a receptor for estrogen exists on the surface of the cell (Watters et al., 1997). Another report, which was based on an investigation using well-characterized antibodies that recognized multiple estrogen receptor epitopes, demonstrated that the estrogen receptor is located at the surface of cell (Pappas et al., 1995). In the present study, we demonstrated that, in the rat hippocampus, membrane-impermeable estradiol activates ERK within several minutes, a period in which estradiol is not considered able to induce estrogen response element-mediated transcription. In addition, these effects were not blocked by the simultaneous administration of tamoxifen and ICI 182780, which are nuclear estrogen receptor antagonists. 17α-Estradiol, which is an isomer of 17β-estradiol and dose little possess bioactivity, did not affect the activity of ERK under our condition (data not shown), suggesting that the activation of ERK by 17β-estradiol is not induced by the interaction of steroids with the lipid components of the cell membrane. These data indicate that the rapid activation of ERK by estradiol in vivo is mediated through a putative membrane-bound es-

trogen receptor in the rat hippocampus. The method used in this study is useful to experiments involving the nervous system in vivo. The physiological role of the rapid activation of ERK by estrogen in vivo presented in this report has not yet been elucidated. More recently, an in vitro study reported that activation of the MAPK pathway by estrogen participates in mediating neuroprotection in primary cortical neurons (Singer et al., 1999). Further studies are required to investigate whether or not pretreatment with estradiol can prevent neuronal cell damage via the ERK cascade in vivo.

Acknowledgements

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