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17-β ESTRADIOL RAPIDLY ENHANCES EXTRACELLULAR SIGNAL-REGULATED KINASE 2 PHOSPHORYLATION IN THE RAT BRAIN

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Abstract—Physiological doses of 17-β Estradiol (E_2) rapidly induce mitogen-activated protein kinase (MAPK) phosphorylation in a variety of cell culture and tissue explant preparations. Rapid MAPK phosphorylation has been implicated as a critical step in estrogen's effects on neuronal activity, gene transcription and neuroprotection. The present series of *in vivo* experiments were designed to determine whether acute administration of estrogen rapidly increased extracellular signal-regulated protein kinase (ERK) 2 phosphorylation. Brains were harvested 20 min after a single i.p. injection of 15 μg/kg of 17-β or 17-α estradiol. Twelve brain structures were micro-dissected, homogenized and processed for Western blotting. E_2-treated rats exhibited a statistically significant increase in ERK2 phosphorylation in the diagonal band of Broca, rostral nucleus accumbens, paraventricular nucleus, arcuate nucleus and anteromedial visual cortex. Administration of the same dose of 17-α estradiol did not enhance ERK phosphorylation in any of the brain regions examined. The *in vivo* data presented here extend previously published *in vitro* data indicating that E_2 rapidly activates MAPK in primary neuronal cultures, explants and cell lines. These data also indicate that MAPK activation is a potential mediator of estrogens effects in some but not all estrogen receptor containing regions of the brain. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: ERK2, rapid signaling, *in vivo*, diagonal band of Broca, nucleus accumbens, arcuate nucleus.

Exposure to the steroid 17-β estradiol (E_2) is associated with a variety of CNS effects including changes in RNA transcription, neuronal morphology, behavior, and cognition. Although E_2 has antioxidant properties at pharmacological doses (Behl, 2002), exposure to physiological E_2 doses activates estrogen receptors (ERs) which initiate transcriptional and/or rapid non-transcriptional mechanisms (see McEwen, 2001 for review). In recent years, ER mediated non-transcriptional signaling has been a subject of intense study. We now know that a variety of signaling mechanisms are rapidly activated in response to estrogen stimulation, such as membrane ER coupling to G-proteins, activation of adenylyl cyclase, protein kinase A (PKA), protein kinase C (PKC; Qiu et al., 2003), src (Migliaccio et al., 1996) phosphorylation of cyclic AMP response element binding protein (CREB; Abraham et al., 2004 1), fos, c-jun (Webb et al., 1995; Zhou and Dorsa, 1994) and mitogen-activated protein kinases (MAPKs; Watters et al., 1997) among others (reviewed in McEwen, 2001; Behl, 2002).

A growing body of literature indicates that E_2 elicits a variety of neuroendocrine and/or behavioral responses, many of which occur within minutes of steroid exposure. E_2 rapidly enhances recognition memory in rats (Luine et al., 2003). E_2 also rapidly regulates nociception in Japanese quail (Evrard and Balthazart, 2004). In the rodent hippocampus, insulin-like growth factor 1 (IGF-1) and E_2 cross-regulate the expression of ER and IGF-1 receptor respectively (reviewed in Cardona-Gomez et al., 2003). Amphetamine (AMPH)-induced rotational behavior and striatal dopamine (DA) release are enhanced by E_2 exposure (Becker, 1990). Given the rapid nature of many of E_2's effects, it is tempting to speculate that non-transcriptional signaling mechanisms are involved in these physiological responses. Thus, it becomes important to identify brain regions that exhibit rapid non-transcriptional signaling pathway activation in response to E_2 exposure.

Although most studies examining E_2-initiated rapid signaling utilize cell lines or tissue explant preparations, there is accumulating evidence that low E_2 doses rapidly (within 30 min) increase MAPK phosphorylation *in vivo*. Kuroki et al. (2000) infused 10 μM bovine serum albumin (BSA)-conjugated E_2 into the left ventricle and observed increased extracellular signal-regulated kinase (ERK) enzymatic activity as well as a rapid (5 min) induction of ERK phosphorylation in the rat hippocampus. The authors subsequently demonstrated that rapid ERK phosphorylation is necessary for E_2-mediated protection against quinolinic acid toxicity in the rat hippocampus (Kuroki et al., 2001). During the preparation of this manuscript, Abraham et al. (2004) demonstrated that E_2 rapidly

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Abbreviations: AMPH, amphetamine; AVPV, anteroventral periventricular nucleus; BFCN, basal forebrain cholinergic neurons; BSA, bovine serum albumin; cPOA, caudal preoptic area; CREB, cyclic AMP response element binding protein; DA, dopamine; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; E_2, 17-β estradiol; E_2-BSA, bovine serum albumin-conjugated estrogen; IGF-1, insulin-like growth factor 1; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; mPOA, medial preoptic area; ovx, ovariectomized, ovariectomy; pCREB, cyclic AMP response element binding protein phosphorylation; PI, protease inhibitor cocktail I; PKA, protein kinase A; PKC, protein kinase C; POA, preoptic area (rostral); PVDF, polyvinylidene difluoride; PVN, paraventricular nucleus of the hypothalamus; p90RSK, p90 ribosomal S6 kinase; TBS-T, Tris-buffered saline–Tween-20; VMH, ventromedial nucleus of the hypothalamus.

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increased the number of phospho-ERK and phospho-CREB immunoreactive cells in various mouse hypothalamic nuclei in a receptor-dependent manner. In light of the increasing relevance of E_2/MAPK signaling, the present studies were designed to determine whether systemic administration of a single dose of E_2 commonly used in neuroendocrine and behavioral studies influences MAPK phosphorylation in 12 rat brain structures known to express ERα and/or ERβ. We present here the first comprehensive examination of estrogen's rapid effects on ERK phosphorylation in the rat brain.

EXPERIMENTAL PROCEDURES

The Oregon Health and Science University institutional animal care and use committee has approved all procedures using animals. The experiments described in this report conformed to guidelines established by the National Institutes of Health for the humane treatment of animals. All efforts were made to minimize the number of animals used and their suffering. Vendor-purchased ovariectomized (ovx) rats (230–260 g; Taconic, Germantown, NY, USA) were maintained *ad libitum* on a phytoestrogen-reduced diet (TEKLAD 16% Protein Rodent Diet; Animal Specialties, Woodburn, OR, USA) for 5–7 days. Rats were also handled and sham injected daily during this period. All experiments were performed using rats that were less than 2 weeks post-ovx. Each rat received a single i.p. injection of vehicle (ethanol/saline 1:4, 0.2 ml), or 15 μg/kg (approximately 3.75 μg per rat) of 17-β or 17-α estradiol (Steraloids, Newport, RI, USA). Twenty minutes later, rats were anesthetized with 0.5–1.0 mg/kg ketamine/xylazine/acepromazine (55.5 mg ketamine+5.5 mg xylazine+1.1 mg acepromazine) and quickly decapitated.

Micro-dissections

Each brain was rapidly removed from the skull and rinsed in ice cold immunoprecipitation (IP) buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 100 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM sodium orthovanadate). One-millimeter sections were made through the chilled brain using a rat brain matrix (200–400 g; Ted Pella Inc., Redding, CA, USA) with double-edged razor blades. Sections were placed in ice-cold IP buffer containing 0.1% Triton X-100 and protease inhibitor cocktail I (PI) (1:50; Calbiochem, San Diego, CA, USA). Brain regions were quickly micro-dissected (Fig. 1b) using a dissecting microscope based on the atlas *Brain Maps: Structure of the Rat Brain* (Swanson, 1992). All of the dissections were limited to the structure of interest with a few exceptions. The diagonal band of Broca was dissected from two 1 mm sections, one of which also contained the vascular organ of the lamina terminalis. The dissection designated "rostral" preoptic area (POA) contained the anteroventral periventricular nucleus (AVPV), and the medial preoptic area (mPOA). The dissected caudal preoptic area (cPOA) region also contained the ventral anterior hypothalamus.

Western blotting

Micro-dissected brain structures were homogenized in microfuge tubes containing IP buffer/1% Triton/PI and centrifuged for 20 min. Supernatants were collected and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Rockford, IL, USA). Protein samples (5 μg) were resolved under denaturing/reducing conditions on pre-cast 4–12% Bis–Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred to a PVDF membrane that was blocked in 5% nonfat-dry milk in Tris-buffered saline–Tween-20 (0.5%) (TBS-T) for 1 h at room temperature. Membranes were incubated in 5% milk/TBS-T containing phospho MAPK E10 monoclonal antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. Membranes were subsequently rinsed in TBS-T and incubated in goat–anti-mouse–HRP secondary antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized using enhanced chemiluminescence (NEN, Boston, MA, USA) on a UVP EpiChemi Darkroom Machine (Upland, CA, USA). Blots were stripped (Re-Blot plus; Chemicon, Temecula, CA, USA), blocked in 5% milk and reprobed with polyclonal ERK2 (C-14; Santa Cruz). Blots were rinsed and incubated in goat–anti-rabbit–HRP secondary antibody (Santa Cruz). Immunoreactive bands were visualized as described above.

Western blot quantification

Densitometry was performed using Laboratory Works software on the UVP EpiChemiDarkroom Machine. Data were quantified as described in Fitzpatrick et al., (2002).

$$V_1 \text{ Density ratio} = \frac{(pERK\ 2 - background)}{(ERK\ 2 - background)}$$

$$\frac{\frac{V_1}{V_{avg}} + \frac{V_2}{V_{avg}} + \cdots \frac{V_n}{V_{avg}}}{V \text{ sample size}} = 1 +/- \text{std error}$$

$$\frac{\frac{E_1}{V_{avg}} + \frac{E_2}{V_{avg}} + \cdots \frac{E_n}{V_{avg}}}{E \text{ sample size}} = \text{Fold change from } V_{avg} +/- \text{std error}$$

A ratio is calculated that is equivalent to phospho-ERK2 density divided by the ERK2 density (after background density values are subtracted). Vehicle-treated rats were normalized to one by dividing the density ratio for each animal (V_1, V_2. . .) by the mean density (V_{avg}) of all vehicle-treated rats on that particular gel. The density ratio of each estrogen (E_1, E_2. . .)-treated animal was also divided by the mean density ratio for vehicle-treated rats from the same gel to obtain the fold change in ERK2 phosphorylation in E_2 or 17-α estradiol-treated rats when compared with vehicle-treated counterparts.

Circulating estrogen levels

Trunk blood was collected from rats at time of decapitation. Circulating estrogen levels were determined using radioimmunoassay reagents from Diagnostics Products Corporation (Los Angeles, CA, USA). The interassay coefficient of variation was less than 8%. This assay was highly specific to E_2 with a 0.02% cross-reactivity to 100 ng of 17-α estradiol.

RESULTS

E_2 rapidly enhances ERK2 phosphorylation in several rat brain structures

Fig. 1a schematically depicts the experimental protocol used in these studies. The effect of treatment on ERK2 phosphorylation was assumed to be terminated once the brain was placed in the ice cold rat brain matrix at 0 °C. Twelve brain structures were micro-dissected under these conditions and examined for changes in ERK phosphorylation (Fig. 1b). Circulating E_2 levels were determined in a subset of animals that received vehicle or E_2 injection. At 20 min post-injection, vehicle-treated rats exhibited circulating E_2 levels that were less than 20 pg/ml while E_2-