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Estrogen receptor-mediated transcription involves the activation of multiple kinase pathways in neuroblastoma cells

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ABSTRACT

While many physiological effects of estrogens (E) are due to regulation of gene transcription by liganded estrogen receptors (ERs), several effects are also mediated, at least in part, by rapid non-genomic actions of E. Though the relative importance of rapid versus genomic effects in the central nervous system is controversial, we showed previously that membrane-limited effects of E, initiated by an estradiol bovine serum albumin conjugate (E2-BSA), could potentiate transcriptional effects of 17β-estradiol from an estrogen response element (ERE)-reporter in neuroblastoma cells. Here, using specific inhibitors and activators in a pharmacological approach, we show that activation of phosphatidylinositol-3-phosphate kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways, dependent on a $G\alpha_q$ coupled receptor signaling are important in this transcriptional potentiation. We further demonstrate, using ERα phospho-deficient mutants, that E2-BSA mediated phosphorylation of ERα is one mechanism to potentiate transcription from an ERE reporter construct. This study provides a possible mechanism by which signaling from the membrane is coupled to transcription in the nucleus, providing an integrated view of hormone signaling in the brain.

1. Introduction

Estrogens are important in reproduction, neuroprotection and cardiovascular function [1–6] by binding the ER [7,8] α and β, which in turn regulate transcription of target genes [9,10]. Transcriptional regulation by estrogens via ERα binding to estrogen response elements (EREs) is a relatively slow genomic mode of action common to all members of the nuclear receptor superfamily [11]. However, alternative signaling pathways that are reminiscent of G-protein coupled signaling pathways also exist in response to estrogens. For example, neurite outgrowth of mouse midbrain dopaminergic neurons is increased by estrogens via protein kinase A (PKA) activation, calcium increase [12,13], and Akt activation [14]. In rat hippocampal neurons, estrogen induces calcium dependent src/MAPK, increasing Bcl-2 mRNA and subsequent neuroprotection against glutamate excitotoxicity [15]. The PI3K pathway, also induced by estrogens, reduces ischemia-induced CA1 cell death [16] and activates PKA and PKC in hypothalamic neurons to inhibit GABA neurotransmission [17], leading to depolarization and increase in excitability. Therefore, non-genomic actions of estrogens in the brain are prominent [18–22] and involve both kinase activation and calcium flux [23,24].

Do the outcomes of non-genomic signaling such as neuroprotection involve transcription? In endothelial cells, PI3K activation by 17β-estradiol (17β-E) increased cyclooxygenase 2 transcription, showing integration of kinase activation to gene transcription [25]. In MCF-7 cells, 17β-E upregulates superoxide dismutase and glutathione peroxidase mRNA via the MAPK pathway [26], and ERα and ERK2 collaborate in regulating gene and proliferation programs in human breast cancer cells [27]. Our previous study using a novel two pulse paradigm of hormone administration in SK-N-BE(2)C neuroblastoma cells, demonstrated that rapid actions initiated by membrane-limited E2-BSA potentiates transcription by 17β-E from an ERE [28]. Using this two pulse paradigm in female rats, Kow et al. showed facilitation of lordosis [29] by E2-BSA. This suggests that an integrated pathway exists in neurons to couple non-genomic signals from the plasma membrane by 17β-E to both transcription and behavior. Since different cell types respond differently to E2-BSA [30], our first objective was to investigate pathways required for E2-BSA potentiation of transcription in neuroblastoma

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cells. We show here that MAP/ERK kinase (MEK) and PI3K signaling, in addition to the PKA and PKC signaling shown previously [28], possibly initiated by a $G\alpha_q$ coupled receptor, are required for E2-BSA to increase transcription from the ERE by 17β-E. We also hypothesized that one mechanism to couple non-genomic actions to genomic actions is the phosphorylation of the ERα. Using ERα phosphorylation deficient mutants where the serines are mutated to alanines, we show that ERα phosphorylation on serines (S) 167 and 118, presumably by activated kinases, provides at least one mechanism to couple the rapid actions of 17β-estradiol initiated at the membrane to transcription in the nucleus.

2. Methods and materials

2.1. Plasmids/constructs/chemicals

The pGL2-TATA-Inr-Luc construct with three EREs upstream of the luciferase reporter [31], the pSG-hERα constructs [32,33] and the ERα phosphorylation mutants (S118A, S167A and S236A) were as described [34]. The dominant $G\alpha_s$ construct was a gift from Dr. Berlot [35], the $G\alpha_q$ dominant negative mutant Q209L/D277N (Clone id:GNA0Q000X0) and the $G\alpha_q$ constitutive mutant Q209L (Clone id:GNA0Q000C0) were from the Guthrie Resource Centre, Sayre, PA. The AcGFP-ERα plasmid was constructed by first cloning the AcGFP cDNA into pCMV-Tag2B (Stratagene, CA), then cloning the full length ERα in a position that is N terminal to the AcGFP protein. The E2-6-BSA (17β-estradiol 6-(carboxymethyl) oxime:BSA) (E2-BSA) (Sigma, MO) in distilled water was filtered as per Stevis et al. to remove free 17β-E [36]. Despite the possibility of 17β-E leaching from E2-BSA [36], we previously showed that filtered 10^{-9} M E-BSA applied for 24 h to these cells did not induce transcription, suggesting that leaching is negligible [37] from E2-BSA in this cell line. The final concentrations of 17β-estradiol in the E2-BSA in distilled water and 17β-E (Sigma, MO) (dissolved in ethanol) used in experiments were 10^{-9} M unless stated otherwise.

2.2. Cell culture and transfections

SK-N-BE(2)C cells, a human neuroblastoma cell line, incubated at 37 C/5% $C0_2$, were plated in Ham's F12:Minimal Essential Media (MEM) (1:1; Cellgro Inc, USA) with 10% fetal bovine serum (FBS) (Bioreclamation, Inc), 100 units/ml penicillin and 50 μg/ml streptomycin in 6-well plates (Falcon) at a density of 0.3×10^6 cells/well and transfected with pGL2-TATA-Inr-Luc (200 ng), pSG-hERα (80 ng), pSV-βgal (80 ng) and pBSKII+ to a total of 400 ng/well, using the Effectene reagent (Qiagen, CA) according to the manufacturer's instructions, 48 h after plating. Transfection efficiencies were routinely 20–30%. Twenty-four hours after transfection, the cells were washed with Dulbecco's phosphate buffered saline (D-PBS), and phenol-red free Ham's F-12: Minimal Essential Media (MEM) (1:1; Cellgro USA), with 5% charcoal dextran stripped FBS (Gemini Biotech) and antibiotics (Cellgro Inc., USA) added to the cells. When mutant constructs were used, transfection is done 24 h after plating and the two-pulse hormone regimen started 48 h after transfection, to allow maximal expression from constructs. *The two pulse paradigm*: After media change, a hormone regimen of two pulses of hormones and/or enzyme inhibitors, separated by a hormone-free interpulse interval was initiated. At the end of the first 20-min pulse, fresh hormone free media was added to cells for the interpulse interval (4 h), after which the 2 h second pulse was initiated. E2-BSA in the first pulse followed by 17β-E2 in the second pulse was designed to limit E actions to the membrane in the first 20 min. Hormones and inhibitors/activators (Calbiochem, MA) ($n = 4$–8 wells/treatment group) were added in either pulse at concentrations shown in the figures. In a modified two-pulse paradigm used only with the phospho-mutants (Fig. 7), the first pulse was 20 min and was followed by a 1-h 2nd pulse, 2 h after the first. In all cases, cells were lysed 24 h after the start of the first pulse with Glo Lysis Buffer and the lysate was assayed for luciferase activity in each sample. This was normalized to βgalactosidase (βgal) activity in the same lysate using the Promega Kit according to the manufacturer's instructions (Promega, WI). Results are expressed (mean ± SEM) are plotted (Graph Pad Software, CA) as fold induction over that in the ethanol and BSA treated group (vehicle controls).

2.3. ELISA and immunocytochemistry (ICC)

Transfected cells ($n = 4$ wells/treatment group) were treated with vehicle or 10^{-9} M 17β-E or E2-BSA for 20 min and lysed using Milliplex Cell Signaling and Lysis Buffers (Millipore, MA). Lysate from 2 wells was pooled; 10 μg total protein, determined by the Bradford assay, was assayed for both phospho-enzyme and total enzyme using the Milliplex MAPmates kit. The phospho-targets, pAkt (Ser 473; #46-601; Millipore, MA) and pERK (Thr 185/Tyr 187; #46-602), were normalized to total Akt and ERK per μg protein. For ICC, cells were plated at a density of 0.4×10^5 cells on poly-D-lysine coated (50 μg/ml) 12 mm coverslips (Thickness 0; Carolina Biologicals Inc., USA) placed in 24 well plates. Cells were transfected using Effectene (Qiagen, CA), with AcGFP tagged-ERα (100 ng/well), 24 h after plating; media was also changed to phenol red free DMEM:F12 and charcoal stripped FBS at this time. 48 h after transfection, cells were treated with vehicle, 10^{-9} M 17β-E or 10^{-8} M E2-BSA for 20 min, fixed with 4% PFA/15 min/RT and blocked with 10% donkey serum in PBS and 0.2% Triton X-100 (PBST). The primary antibody H-184 (Santa Cruz sc-7207; 1:300 in PBST), targeted to the N-terminal of ERα, was followed by donkey anti-rabbit 594 (Jackson Immunoresearch, 1:600) in PBST. Cells were imaged at 200× magnification on Olympus IX71 (Hamamatsu Camera; 0.1 s exposure) using HC Image Software, after mounting with DAPI-Vectashield (VectorLabs, CA). Exposure time was determined after comparison to transfected cells treated solely with secondary antibody.

2.4. Real time PCR

Total RNA from transfected cells treated with vehicle or 10^{-9} M 17β-E or E2-BSA ($n = 4$–8/treatment group) for 20 min or to a two pulse paradigm ($n = 7$–8/treatment group) was isolated with the RNAeasy Kit (Qiagen, CA). The ERα forward primer is 5′AGGGAAGCTCCTATTTGCTCC3′ and reverse primer is 5′CGGTGGATGTGGTCCTTCTCT3′. 25 ng of total transcribed (RT Promega Kit) RNA was amplified using Fast SYBR Green Master Mix (Applied Biosystems, CA) on an Applied Biosystems 7900HT Fast Real-Time PCR System, at the Penn State Genomics Core Facility, University Park, PA. After an initial denaturation at 95 °C for 10 s, 40 cycles of 95 °C for 5 s (denaturation), 60 °C for 15 s (annealing) and 72 °C for 10 s (extension) were performed. ERα in each sample was normalized to cyclophilin. Abundance of normalized ERα mRNAs, relative to control treatment, was calculated using the $2^{-\Delta\Delta CT}$ method [38].

2.5. Western blotting

Transfected cells ($n = 4$/treatment group) were treated with 10^{-8} M E2-BSA, 10^{-9} M 17β-E or vehicle for 10, 20 or 40 min, rinsed in PBS and cold RIPA lysis buffer (Boston Bioproducts, MA) containing 1% protease and 2% phosphatase inhibitor cocktail (Sigma–Aldrich, MO) added, centrifuged at 13,000 rpm/10 min/4 °C and the supernatant stored at −20 °C until use. After protein estimation with Lowry reagent (DC Protein Assay Kit, BioRad, CA), 20 μg total protein was denatured for 7 min/100 °C in Laemelli buffer and loaded into SDS-PAGE gels (10% separating). Protein