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17β-Estradiol induces ERβ up-regulation via p38/MAPK activation in colon cancer cells

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Abstract

Estrogen receptors (ERα and ERβ) mediate opposite functions on cancer growth induced by 17β-estradiol (E2). E2 binding to ERα induces a cancer promoting response, whereas E2 binding to ERβ exerts a protective action against cancer growth. Moreover, E2 can diversely modulate the ERα and ERβ levels intensifying or decreasing their actions in target tissues. Only molecular mechanisms at the root of E2 ability to down-regulate the ERα levels are known. Here, we report the first molecular mechanism underlying E2-induced ERβ up-regulation in DLD-1 colon cancer cells. E2 induces a short term (2 and 4 h after stimulation) translation of ERβ mRNA followed by a late (24 h after stimulation) enhanced transcription. Both processes required the E2-induced persistent and palmitoylation-dependent p38/MAPK activation. Overall, our data suggest a finely tuned control exerted by rapid signals on different cellular molecular events important for the protective effects of E2 against colon cancer growth.

Keywords: 17β-Estradiol; Estrogen receptor β; Non-genomic signals; p38/MAPK; Colon cancer cells

Besides their classical role in sexual development and function, estrogen receptors (ERα and ERβ) mediate 17β-estradiol (E2) functions in many non-reproductive tissues [1]. Knockout mice analysis showed that ERα and ERβ have markedly different tissue distribution [2] and mediate diverse E2 effects [3,4]. Evidences accumulated suggesting ERα as mediator of the E2-induced cancer promoting response in several target cells [5–7], whereas the loss of ERβ (the predominant ER subtype expressed in human colon) is associated with advanced stages of colon cancer and tumor cell de-differentiation, thus suggesting its protective role in colon cancerogenesis [8–13].

In cancer progression, the analysis of the various roles played by ER isoforms, is further complicated by the fact that E2 can modulate ERα and ERβ expression intensifying or decreasing their actions in target tissues. E2 administration to ovariectomized rats reduces the uterine levels of ERα by approximately 60% [14] and E2 exposure down-regulates the steady-state level of ERα in MCF-7 cells, as reflected by the protein half-life decrease [15,16]. The potential E2-dependent regulation of ERβ levels, which can play a fundamental role in the control of cell proliferation, is less known although a time-dependent E2-induced increase in ERβ mRNA has been observed in the human cell lines of breast cancer T47D [4,17], prostate cancer DU145 [18], and colon cancer DLD-1 [13].

E2 exerts its powerful effects on cell physiology mainly by regulating gene transcription, even if post-transcriptional regulation of gene expression (including mRNA translation) represents a mechanism to control and modify the flow of genetic information into the proteome [19]. This latter system is commonly used by E2 to auto-regulate the expression of the mRNAs that encode its own receptor proteins [20], but the molecular mechanisms involved are unknown.

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The discovery of membrane-associated ER pools capable of eliciting both genomic and non-genomic responses [1,21–24] have led to a deep re-evaluation of the mechanisms underlying E2 action. Recent investigations have revealed that ERs are highly mobile proteins continuously shuttling between the plasma membrane and other cellular compartments [22,25,26]. Ligand- and/or protein-induced ER conformational changes regulate such movements leading to specific responses. Receptor anchorage at specific sites as well changes in receptor levels could be determined by these shuttling mechanisms [25]. Indeed, we previously demonstrated that in human DLD-1 colon cancer cells, ERβ palmitoylation was necessary for its localization at the plasma membrane and to associate with caveolin-1 and the p38 member of MAPK family [13]. Moreover, the palmitoyl acyl transferase (PAT) inhibitor 2-bromohexadecanoic acid (2-Br) blocked the ability of ERβ–E2 complex to activate p38 impairing the receptor-dependent activation of downstream pro-apoptotic cascade (i.e., caspase-3 activation and PARP cleavage) [13].

In order to verify the hypothesis that E2 might triggers such rapid, non-genomic action by the up-regulation of ERβ levels, we used the DLD-1 model system, devoid of ERα [13], and demonstrated that E2 rapidly increases ERβ levels via the palmitoylation-dependent persistent p38/MAPK activation. In turn, p38/MAPK yields both rapid translational and slow transcriptional mechanisms able to maintain high level of ERβ important for the E2 anti-proliferative effects.

Materials and methods

Reagents. E2, gentamicin, penicillin, RPMI1640 (without phenol red), charcoal-stripped fetal calf serum, the PAT inhibitor 2-bromohexadecanoic acid (2-Br), actinomycin, and cycloheximide were purchased from Sigma–Aldrich (St. Louis, MO, USA). The p38/MAPK inhibitor SB-203580 (SB) and the proteasome inhibitor MG-132 were obtained from Calbiochem (San Diego, CA, USA). The ER inhibitor ICI-182780 (ICI) was obtained from Tocris (Ballwin, MO, USA). Bradford Protein Assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The anti-β-actin and the anti-ERβ L20 (C-terminus) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-ERβ-14C8 (N-terminus) antibody was purchased from Genetex (San Antonio, TX, USA). The anti-phospho-p38 and anti-p38 antibodies were obtained from New England Biolabs (Beverly, MA, USA). The ECL chemiluminescence reagent for Western blot was obtained from Amersham Biosciences (Little Chalfont, UK).

All the other products were from Sigma–Aldrich (St. Louis, MO, USA). Analytical or reagent grade products, without further purification, were used.

Cell culture and counting. Human DLD-1 colon adenocarcinoma cells [11] were routinely grown in air containing 5% CO_2 in modified phenol red-free RPMI1640 medium, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml).

DLD-1 cells were grown to ~70% confluence in 6-well plates and then stimulated. After treatment (10 nM E2, 1 μM ICI-182780, 5 μM SB-203580, 10 μM 2-Br), cells were harvested by trypsinization, centrifuged, stained with Trypan blue solution, and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate.

RNA isolation and quantitative RT-PCR analysis (qRT-PCR). The sequences for gene-specific forward and reverse primers were designed using the OligoPerfect™ Designer software program (Invitrogen, Carlsbad, CA, USA). The following primers were used: for human ERα (ESR1/NR3A1, GenBank Accession No. AY425004), 5′-TCCTAGCAGGGAGATGAGGA-3′ (forward) and 5′-CCTTTATGGCCAGCAATCAT-3′ (reverse), for human ERβ (ESR2/NR3A2, GenBank Accession No. AY785359), 5′-GGCGCGATCTTGGCTCAC-3′ (forward) and 5′-TGGCTGGACGTGGTGGCA-3′ (reverse), and for β-actin (GenBank Accession No. X00351), 5′-AGAAGGATTCCTATGTGGGCG-3′ (forward) and 5′-CATGTCGTCCCAGTTGGTGAC-3′ (reverse).

Total RNA was extracted from DLD-1 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To determine hERβ gene expression levels (as well to confirm the absence of hERα), cDNA synthesis and qPCR were performed using a one-step qRT-PCR kit ("SuperScript™ III Platinum®—SYBR® Green One-Step" kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis and qPCR were carried out in a ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as follows: first strand cDNA synthesis was performed at 50 °C for 5 min, followed by an automatic hot-start *Taq* DNA Polymerase activation step at 95 °C for 5 min, and then by 25 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 40 °C for 1 min. Gene expression was verified by 2% agarose gel electrophoresis. Each sample was tested in duplicate and the experiment repeated 4 times.

Electrophoresis and immunoblotting. After treatments, cells were lysed and solubilized [in 0.125 M Tris, pH 6.8, containing 10% (w/v) SDS, 1.0 mM phenylmethylsulfonyl fluoride, and 5.0 μg/ml leupeptin] and finally boiled for 2 min. Total proteins were quantified using the Bradford Protein Assay. Solubilized proteins (20 μg) were electrophoretically resolved by 10% SDS–PAGE (100 V, 1 h, 24 °C) and then transferred to nitrocellulose (100 V, 45 min, 4 °C). The nitrocellulose membrane was treated with 3% (w/v) BSA in 138.0 mM NaCl, 25.0 mM Tris, pH 8.0, at 24 °C for 1 h and then probed overnight at 4 °C with either anti-phospho-p38 or anti-ERβ antibodies. The nitrocellulose membrane was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL, USA) for 10 min at room temperature and then probed with either anti-p38 or anti-β-actin antibodies. Antibody reactions were visualized by chemiluminescence with the Western blotting detection reagent.

Results

As already reported, human DLD-1 colon adenocarcinoma cells contain only one ERβ isoform corresponding to a 54 kDa protein [11,13]. The time course of DLD-1 stimulation by E2 (10 nM) showed that ERβ protein levels increased from 1 to 24 h (Fig. 1A). The E2-induced ERβ increase was ER-dependent since it was prevented by a pre-treatment with the pure antiestrogen ICI (Fig. 1A). The dose-dependent effect showed that, after 2 h of stimulation, 1 nM E2 induces ERβ up-regulation with a peak at 10 nM. No further increase was detectable at higher E2 concentration (data not shown). To verify that the E2-induced ERβ up-regulation was independent of a block in protein degradation, DLD-1 was stimulated with 10 nM E2 in the presence or absence of the proteasome inhibitor MG-132 (15 μM). E2 or MG-132 stimulation induced a similar increase of ERβ levels, while the co-treatment was additive (Fig. 1B). This data indicates that the E2 effect is not mediated by a block of proteasomal protein degradation.

We next evaluated the mechanisms underlying this E2 effect assessing the involvement of E2-induced genomic