Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Estrogen receptor-alpha 36 mediates the anti-apoptotic effect of estradiol in triple negative breast cancer cells via a membrane-associated mechanism

Reyhaan A. Chaudhri [a,b], Agreen Hadadi [a], Kirill S. Lobachev [a], Zvi Schwartz [c], Barbara D. Boyan [b,c,*]

[a] The School of Biology and the Petit Institute for Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, GA, USA
[b] Atlanta Clinical and Translational Science Institute, Emory University, Atlanta, GA, USA
[c] College of Engineering, Virginia Commonwealth University, Richmond, VA, USA

ARTICLE INFO

Article history:
Received 1 March 2014
Received in revised form 28 July 2014
Accepted 30 July 2014
Available online xxxx

Keywords:
Estrogen
Breast cancer
Taxol
Estrogen receptor-alpha 36
Plasma membrane
Anti-apoptosis

ABSTRACT

17β-Estradiol can promote the growth and development of several estrogen receptor (ER)-negative breast cancers. The effects are rapid and non-genomic, suggesting that a membrane-associated ER is involved. ERα36 has been shown to mediate rapid, non-genomic, membrane-associated effects of 17β-estradiol in several cancer cell lines, including triple negative HCC38 breast cancer cells. Moreover, the effect is anti-apoptotic. The aim of this study was to determine if ERα36 mediates this anti-apoptotic effect, and to elucidate the mechanism involved. Taxol was used to induce apoptosis in HCC38 cells, and the effect of 17β-estradiol pre-treatment was determined. Antibodies to ERα36, signal pathway inhibitors, ERα36 deletion mutants, and ERα36-silencing were used prior to these treatments to determine the role of ERα36 in these effects and to determine which signaling molecules were involved. We found that the anti-apoptotic effect of 17β-estradiol in HCC38 breast cancer cells is in fact mediated by membrane-associated ERα36. We also showed that this signaling occurs through a pathway that requires PLD, LPA, and PI3K; Gαs and calcium signaling may also be involved. In addition, dynamic palmitoylation is required for the membrane-associated effect of 17β-estradiol. Exon 9 of ERα36, a unique exon to ERα36 not found in other identified splice variants of ERα with previously unknown function, is necessary for these effects. This study provides a working model for a mechanism by which estradiol promotes anti-apoptosis through membrane-associated ERα36, suggesting that ERα36 may be a potential membrane target for drug design against breast cancer, particularly triple negative breast cancer.

1. Introduction

Although the 5-year survival for patients diagnosed in the early stages of breast cancer exceeds 90%, survival in patients with distant metastasis drops below 25% indicating very poor prognosis for these individuals [1]. While an actual cure for breast cancer is elusive, novel approaches to diagnosis and treatment can help to reduce mortality and allow patients, specifically those with more advanced stage cancer, to live normal lives.

The progression of cancer is a dynamic process that begins with primary tumor growth, depending on cancer cell proliferation simultaneously with the ability of cancer cells to evade apoptosis [2]. In some cases, aggressive cancer cells can evade apoptosis even in the presence of radiotherapy and chemotherapeutic drugs that are used to target these cells and specifically induce apoptosis [3,4]. Current approaches to treatment have evolved to combination therapy, usually beginning with surgery and/or targeted radiotherapy, followed by adjunctive chemotherapy [5–7]. Taxol, a commonly used chemotherapeutic drug, induces apoptosis in cells by inhibiting mitosis [8,9]. Currently, taxol is synthetically prepared and various formulations and carriers are being developed to increase the effectiveness of the drug [10]. Moreover, the main problem with drugs such as taxol is that they do not only target cancer cells, but can also induce apoptosis in normal cells [11,12]. This necessitates current approaches of targeted therapy, by which newer targets are being discovered that are either unique or highly upregulated in cancer cells.

Estrogen receptors (ERs) play a major role in classification, diagnosis, and treatment of breast cancer [13–15]. Patients who have hormone responsive or ER-positive tumors are expected to take the ER-antagonist tamoxifen continuously for 10 years following initial treatment [16]. We previously showed that tamoxifen could block the stimulatory effect of 17b-estradiol (E_2) in not only ER-positive breast cancer cells but also ER-negative breast cancer cells [17]. Traditional inhibitors of ERs, such as ICI 182,780, and diethylsilbesterol, a potent synthetic agonist for ERα, did not block E_2-induced cell proliferation

* Corresponding author at: School of Engineering, Virginia Commonwealth University, 601 West Main Street, P.O. Box 843068, Richmond, VA 23284-3068, USA. Tel.: +1 804 828 0190; fax: +1 804 828 9866.
E-mail address: bboyan@vcu.edu (B.D. Boyan).

http://dx.doi.org/10.1016/j.bbamcr.2014.07.019
0167-4889/

or protein kinase C (PKC) activity in the ER-negative cells, nor did traditional antibodies to ERα and ERβ [17].

Membrane-associated E_2 signaling also elicits anti-apoptotic effects against taxol, leading to an aggressive cancer phenotype [3,18]. We recently showed that ERα36, an alternatively spliced variant to traditional ERα, is responsible for the membrane-mediated effect of E_2 in breast cancer cells that promote cell survivability [18,19]. The mRNA of ERα36 lacks the first exon found in traditional ERα, ERα66, as well as exons 7 and 8 [20]. This results in a truncated form of ERα that does not contain the transcriptional activation domains AF1 or AF2 and a truncated ligand-binding domain; however, ERα36 still exhibits ligand-dependent effects of E_2 [20–22]. In addition, ERα36 contains a novel exon at the C-terminus known as exon 9. This exon contains 27 amino acids of unknown function, but it is hypothesized to contain myristoylation or palmitoylation-specific sequences [20,23], which would help to explain any membrane-associated effects mediated by ERα36. This study investigated a mechanism by which ERα36 prevents taxol-induced apoptosis. We hypothesized that ligand-dependent activation of ERα36 induces receptor-dependent inhibition of signaling cascades associated with anti-apoptosis.

2. Materials and methods

2.1. Reagents

Triple negative HCC38 human breast cancer cells, which we previously showed to also be negative for the ERα splice variants, ERα66 and ERα46, but positive for ERα36 [18], and human embryonic kidney HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Roswell Park Memorial Institute 1640 medium (RPMI 1640) was purchased from Invitrogen (Grand Island, NY). Charcoal/dextran-filtered fetal bovine serum (FBS) was purchased from Gemini Bioproducts (Sacramento, CA). E_2 enantiomer (Ent-E_2) was kindly provided as a gift from Dr. Douglas Covey (Washington University, St. Louis, MO) and has been described previously [24]. E_2, E_2-BSA, taxol, 2-hydroxymyristic acid (HMA), 2-bromohexadecanoic acid (2-bromopalmitate, 2-BP), and tunicamycin (Tm) were purchased from Sigma (St. Louis, MO). Cycloheximide (CHM), Wortmannin, D609, U73122, LY294002, thapsigargin, pertussis toxin (PTX), and cholera toxin (CTX) were purchased from EMD Chemicals (Gibbstown, NJ). VPC32183S and lysophosphatidic acid (LPA) were purchased from Avanti Polar Lipids (Alabaster, AL). Protein content of samples was measured using the Macro BCA reagent kit from Pierce/Thermo Scientific (Rockford, IL). Polyclonal ERα36 antibodies against the unique C-terminal 27 amino acids were generated by Cell Applications Inc. (San Diego, CA). A monoclonal anti-ERα antibody that recognizes the three ERα splice variants ERα66, ERα46, and ERα36 was purchased from Abcam (San Francisco, CA). 740 Y-P and the Titertacs TUNEL assay were purchased from R&D Systems (Minneapolis, MN). Goat anti-rabbit horseradish peroxidase (HRP) and goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). Bax and Bcl2 primers were purchased from Eurofins MWG Operon (Huntsville, AL). The cytochrome C apoptosis assay kit was purchased from MBL International (Woburn, MA). The Amplex Red Phospholipase D (PLD) Assay kit was purchased from Life Technologies (Grand Island, NY). Caspase-3 activity was measured using the CaspAce Assay system from Promega (Madison, WI). ERα36 overexpression plasmids were purchased from Chi Scientific (Maynard, MA). Polyfect transfection reagent was obtained from Qiagen (Germantown, MD).

2.2. Cell culture

HCC38 cells and HEK293 cells were cultured in RPMI 1640-based media or DMEM, respectively, as specified by the ATCC containing 10% charcoal/dextran-filtered FBS and lacking phenol red, which can mimic the effects of E_2 at low levels [25,26]. Specific modifications for each experimental question are described below. For all experimental treatments, the solvent used according to preparation instructions by the manufacturer of each reagent was used in equivalent amounts as a treatment vehicle in all controls.

2.3. Apoptotic effect of taxol in HCC38 cells

The experimental design for this study was based on the ability of E_2 to block the apoptotic effects of taxol. Initial experiments were performed to establish the effect of taxol on HCC38 cells. 24 h after plating, HCC38 cells were treated with increasing concentrations of taxol (5, 10, 20 μM) for 4 h, after which caspase-3 activity was measured using an assay kit according to the manufacturer's directions. To confirm that the effects of taxol were apoptotic, as caspase-3 activity is implicated in the terminal differentiation of some cell types [27–30], HCC38 cells were treated with 20 μM taxol for 12 h and BAX/BCL2 mRNA levels were determined and cytochrome C translocation from the mitochondria to the cytosol was examined by the cytochrome C apoptosis assay kit from MBL International according to the manufacturer's instructions. In addition, 24 h after treatment with taxol, apoptosis-associated DNA-fragmentation was determined using a TUNEL assay kit as per the manufacturer's directions.

2.4. Requirement for a receptor-mediated membrane-associated mechanism

E_2 conjugated to bovine serum albumin (E_2-BSA), which cannot cross the plasma membrane (PM) [31–33], was used to verify that the anti-apoptotic effect of E_2 was via a membrane-mediated mechanism. E_2-BSA has previously been shown to have similar effects to E_2 and can interact with ERs. BSA conjugation prevents E_2 from crossing the PM, and therefore, E_2-BSA effects can be attributed to either membrane receptor effects or alterations in membrane fluidity due to the hydrophobic nature of E_2-BSA [17,31,32,34]. To address the possibility that E_2's effect is due to a non-specific interaction with the PM, cells were also treated with the E_2 enantiomer, Ent-E_2 [24]. While Ent-E_2 has the same chemical structure as E_2 as its enantiomer, it cannot directly interact with ERs, and therefore, any effects caused by Ent-E_2 could be attributed to its direct effect on membrane fluidity, as it possesses the same hydrophobic properties of E_2.

PLD activity was determined as an outcome measure, based on our previous observation that the anti-apoptotic effect of the vitamin D3 metabolite 24R,25-dihydroxyvitamin-D3 (24,25(OH)2D3) occurs through activation of PLD [35]. Subconfluent cultures of HCC38 cells in 24-well tissue culture polystyrene (TCPS) plates were treated with E_2 or Ent-E_2. Also, prior to E_2 treatment, a 15 minute pretreatment of cells with polyclonal ERα36 specific antibodies (1:500 dilution) was performed to block the membrane receptor in order to determine if the effect of E_2 was through membrane-associated ERα36. While antibodies cannot enter the cells, any inhibition of E_2's effect in the presence of antibody could be attributed with E_2's direct interaction with membrane-associated ERα36. Following the 15 minute antibody pretreatment, and a 30 minute E_2 treatment, samples were harvested and assayed for PLD activity using the Amplex Red PLD assay from Life Technologies according to the manufacturer's instructions.

2.5. ERα36 silencing, overexpression, and mutation

In order to confirm the role of ERα36 in the anti-apoptotic effect of E_2, HCC38 cells were transiently transfected with an ERα36 shRNA expression plasmid in order to transiently knockdown ERα36. The shRNA expression plasmid was produced by cloning a microRNA specific antisense target sequence for the 3′UTR of ERα36 cDNA using the DNA oligonucleotides, 5′-GGATCCCATGCCAATAGGTACTGAA-TTGATATCCGTTCAGT ACCTATTGGCATTTTTTTCCAAAAGCTT-3′, and was prepared by Sigma-Aldrich using their Mission shRNA purified plasmid expression system. HCC38 cells were seeded at a density of 1.25×10^5 cells/cm^2 in tissue

Please cite this article as: R.A. Chaudhri, et al., Estrogen receptor-alpha 36 mediates the anti-apoptotic effect of estradiol in triple negative breast cancer cells via..., Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamcr.2014.07.019