Comparative Biochemistry and Physiology, Part D 19 (2016) 159–173

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

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

Differential recruitment of co-regulatory proteins to the human estrogen receptor 1 in response to xenoestrogens☆

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ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form 11 April 2016
Accepted 17 April 2016
Available online 20 April 2016

Keywords:
Estrogen
Estrogen receptor
Xenoestrogens
Fluorescence polarization
Time resolved fluorescence resonance energy transfer
Steroid receptor co-regulator, co-immunoprecipitation, proteomics

ABSTRACT

The diverse biological effects of xenoestrogens may be explained by their ability to differentially recruit co-regulatory proteins to the estrogen receptor (ER). We employed high-throughput receptor affinity binding and co-regulatory protein recruitment screening assays based on fluorescence polarization and time resolved florescence resonance energy transfer (TR-FRET), respectively, to assess xenoestrogen-specific binding and co-regulatory protein recruitment to the ER. Then we used a functional proteomic assay based on co-immunoprecipitation of ER-bound proteins to isolate and identify intact co-regulatory proteins recruited to a ligand-activated ER. Through these approaches, we revealed differential binding affinity of bisphenol-A (BPA) and genistein (GEN) to the human ERα (ESR1) and ligand-dependent recruitment of SRC-1 and SRC-3 peptides. Recruitment profiles were variable for each ligand and in some cases were distinct compared to 17β-estradiol (E2). For example, E2 and GEN recruited both SRC-1 and -3 peptides whereas BPA recruited only SRC-1 peptides. Results of the functional proteomic assay showed differential recruitment between ligands where E2 recruited the greatest number of proteins followed by BPA then GEN. A number of proteins share previously identified relationships with ESR1 as determined by STRING analysis. Although there was limited overlap in proteins identified between treatments, all ligands recruited proteins involved in cell growth as determined by subnetwork enrichment analysis ($p < 0.05$). A comparative, *in silico* analysis revealed that fewer interactions exist between zebrafish (*Danio rerio*) esr1 and zebrafish orthologs of proteins identified in our functional proteomic analysis. Taken together these results identify recruitment of known and previously unknown co-regulatory proteins to ESR1 and highlight new methods to assay recruitment of low abundant and intact, endogenous co-regulatory proteins to ESR1 or other nuclear receptors, in both human and aquatic species.

1. Introduction

Estrogen (E2) is a steroid hormone that controls a diverse array of normal biological processes in vertebrates but is also implicated in reproductive and non-reproductive disease (Dahlman-Wright et al., 2006). Classically, E2 exerts its effects through nuclear estrogen receptors (ERs), which are ligand-dependent transcription factors that regulate gene expression through a complex network of protein–protein and protein–DNA interactions (Gruber et al., 2002). Upon ligand induction, the ER undergoes a series of regulatory modulations, including dimerization, nuclear translocation, and recruitment of co-regulatory proteins to form a macromolecular transcriptional complex. This complex consists of co-activators and/or co-repressors that activate or repress downstream gene targets in response to agonists and antagonists, respectively (Gruber et al., 2002).

It has been hypothesized that differential association of the ERs with co-regulatory proteins directs activation at discrete promoter elements, leading to selectivity in downstream gene expression (Shibata et al., 1997; Shang and Brown, 2002). This phenomenon came to be known as the 'co-activator hypothesis' which describes how the same ligand can manifest different biological activities in discrete tissues within the same animal (McDonnell, 2005). For example, while TAM acts as an ER antagonist in human breast tissue, it acts as an ER agonist in other tissues (McDonnell et al., 2002). This further led to the realization that structurally diverse ER ligands could induce specific structural changes in the conformation of ERs, which lead to variations in co-regulator protein affinity for the receptor, thus, impacting downstream gene activation or repression. (McDonnell et al., 2002).

To date, over 30 ER co-regulatory proteins have been identified in mammalian systems which interact with ER transcriptional complex

☆ This paper is a contribution to the Special Issue on Environmental Omics and Toxicology.

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http://dx.doi.org/10.1016/j.cbd.2016.04.003
1744-117X/

machinery in response to E2 and alternate ligands. The most well-characterized ER complex members are those that comprise the p160 family, including steroid receptor co-activators, SRC-1 (NCoA-1), SRC-2 (TIF2, GRIP-1), and SRC-3 (AIB1, ACTR, p/CIP, RAC3, TRAM-1) (Karmakar et al., 2009). SRC-1, has demonstrated direct binding of specific peptides to the ER in response to E2 through yeast two-hybrid and phage ELISA assays (Hall and Korach, 2002; Lee et al., 2002). Interactions between ER and SRCs have also been suggested in activation of the receptor by employing siRNA strategies as loss of SRC-1 or -3 alters E2-driven cellular processes in human astrocytoma cells (González-Arenas et al., 2012). The family of p160 proteins are also highly expressed in a number of tumors and have been proposed to account for resistance to therapeutics such as aromatase inhibitors or TAM (Tikkanen et al., 2000b; Lauritsen et al., 2002; Xu and Li, 2003; Shao et al., 2004; Labhart et al., 2005; Karmakar et al., 2009; McBryan et al., 2012), highlighting their importance in disease development and treatment regimes.

While the interaction of co-accessory proteins with the ER in response to the endogenous ligand E2 has been rigorously examined, modulation by environmentally-relevant chemicals has yet to be explored in-depth. Hormonally active agents compose a diverse class of natural and synthetic compounds (Kuiper et al., 1998a; Sonnenschein and Soto, 1998; Okubo et al., 2004), and can disrupt steroid signaling in organisms through a variety of mechanisms. For example, xenoestrogens alter E2 signaling by modulation of endogenous steroid synthesis and/or direct modulation of the ERs through agonist or antagonistic means (Shanle and Xu, 2010). Many of these xenoestrogens have been causally linked to adverse health outcomes in target organisms when exposed under environmental conditions (Bergman et al., 2012).

Differential recruitment of co-regulatory proteins represents an additional plausible mechanism for endocrine disruption in humans and other species exposed to xenoestrogens. To date, research on the ligand-dependence of co-regulatory protein recruitment to ERs has focused primarily on therapeutic SERMs that have been used in recent years for the treatment of ER-positive breast cancers. These studies indicated that structurally diverse ligands can drive differential recruitment of co-regulatory proteins within a single cellular context. While most of the investigations of ER-co-regulatory recruitment have focused on the well-studied p160 family members (SRCs) in response to E2, only a handful of studies have reported interactions of these proteins with the receptor in response to binding of ER by the xenoestrogens genistein (GEN), diethylstilbestrol, (DES), bisphenol-A (BPA), and nonylphenol (NP). These targeted analyses were performed primarily by employing glutathione S-transferase (GST) pull-down, combinatorial phage display, yeast two hybrid, and surface plasmon resonance assays (Nishikawa et al., 1999; Paige et al., 1999; Kraichely et al., 2000; Parker et al., 2000; Routledge et al., 2000; Wong et al., 2001), although there is growing interest in employing non-targeted methods to evaluate global co-regulatory protein recruitment.

Based on the current gaps in knowledge, the objectives of our study were to investigate xenoestrogen-induced activation of the human estrogen receptor α (hERα, also known as ESR1) using a suite of *in vitro*, high-throughput real-time receptor binding (fluorescent polarization) assays, to investigate the potential for ligand-specific co-regulatory protein recruitment by SRC-1 and SRC-3 recruitment assays (time resolved fluorescent resonance energy transfer, TR-FRET), and to identify intact co-regulatory proteins bound to endogenous E2- and xenoestrogen-activated ESR1 transcriptional complexes using a co-immunoprecipitation method. Finally, we sought to take a comparative approach to both highlight the utility of our methods in the assessment of differential co-regulatory protein recruitment to nuclear receptors in aquatic species and to compare co-regulatory protein recruitment between human ESR1 and zebrafish (*Danio rerio*) esr1 in response to E2 binding as ER signaling pathways are moderately conserved across species (Lam et al., 2011).

2. Materials and methods

2.1. Mammalian cell culture

MCF-7 cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured in phenol-red free Eagle's Minimum Essential Medium (MEM, Corning cellgro, 17305-CV) supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1% penicillin–streptomycin (Hyclone, SV30010), 1.5 g/L sodium bicarbonate (Corning cellgro, 25-035-CL), 1% L-glutamine (25-005-CI), and 10% fetal bovine serum (Corning cellgro, 35-010-CV). Human Embryonic Kidney 293 (HEK293) cells (ATCC) were cultured in phenol-red free Dulbecco's Modified Essential Medium (DMEM, Corning cellgro, 17-205-CV) supplemented with 1% L-glutamine, 1% penicillin–streptomycin, and 10% fetal bovine serum.

2.2. Fluorescence polarization ligand binding assay

Fluorescence polarization (FP) was first proposed byAdamczyk et al. (2002)) and offers significant advantages compared to conventional radioligand binding protocols. Unlike radioligand-based assays, the FP assay is performed in a homogeneous format that relies on displacement of a fluorescent-labeled estrogen probe from free-solution ER in order to calculate relative binding affinities of ligands. All ligands (17β-estradiol (E2, Sigma, 2578), 4-OH-tamoxifen (TAM, Sigma, T5648), Genistein (GEN, Sigma, G6649), and bisphenol-A (BPA, supplied by NIEHS)) were prepared as concentrated stocks in DMSO and further diluted into binding buffer (10 mM Tris–HCl pH 7.4 (Sigma, 154,563), 50 mM KCl (Sigma, P5405), 10% glycerol (Sigma, G5516), 0.1 mM DTT (Promega, V3151), 0.02% sodium azide (Sigma, S2002), 1 μg/mL bovine gamma globulin (BGG, Sigma, G5009), 0.2% CHAPS (Sigma, C9426)). The pET-32b vector (Millipore, 69016) containing human estrogen receptor alpha ligand binding domain (ESR1-LBD, a gift of Marc Ruff, L'Institut de génétique et de biologie moléculaire et cellulaire (IGBMC), Strasbourg, France), was expressed in origami cells (Millipore) and purified by HPLC (Agilent 1100). Serial dilutions of ligands were prepared in triplicate by adding 200 μL of the working ligand stocks to the first well of each row in a 96-well plate and transferring 100 μL to each additional well containing buffer. Purified ESR1-LBD was diluted and added to each well to give a final ER concentration of 10 nM. Finally, the fluorescent probe (F-E_1), an estrone conjugate coupled to fluorescein-5-thiosemicarbazide (FTSC), was added to give a final concentration of 0.5 nM. The plate was incubated at room temperature in the dark for 1 h prior to analysis. Fluorescence polarization (FP) was measured on a Biotek Synergy H1 spectrophotometer using an excitation wavelength of 485 nm and emission wavelength of 525 nm. FP was converted to percent inhibition ($I_{\%} = (A_0 - A) / (A_0 - A_{100}) * 100$) where A = absorbance and plotted against concentration of ligand using SigmaPlot 11 (Systat Software, Inc., San Jose, CA). Curves were fit by transforming the x-axis to a logarithmic scale and applying a nonlinear regression curve using a sigmoidal-dose response with variable slope to obtain IC_{50} values for the corresponding ligands in SigmaPlot 11.

2.3. Time-resolved fluorescence resonance energy transfer (TR-FRET)

To elucidate the mechanism of xenoestrogen action in recruiting co-activators SRC-1 and SRC-3, we utilized a TR-FRET assay. In this assay, compounds were measured for their ability to recruit model peptides derived from distinct adapter regions of the human SRC-1 and SRC-3 proteins to the ER-ligand complex. The peptides employed in the assay each contain an LXXLL motif and occur in the receptor interacting domain (RID) of the co-regulatory protein. One additional LXXLL peptide for SRC-1 is present in the C-terminal region of the protein (Fig. 2).The Lanthascreen estrogen receptor co-activator kit was purchased from Invitrogen Corporation (A15885), and each assay was