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Unliganded estrogen receptor alpha regulates vascular cell function and gene expression

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ABSTRACT

The unliganded form of the estrogen receptor is generally thought to be inactive. Our prior studies, however, suggested that unliganded estrogen receptor alpha (ERα) exacerbates adverse vascular injury responses in mice. Here, we show that the presence of unliganded ERα decreases vascular endothelial cell (EC) migration and proliferation, increases smooth muscle cell (SMC) proliferation, and increases inflammatory responses in cultured ECs and SMCs. Unliganded ERα also regulates many genes in vascular ECs and mouse aorta. Activation of ERα by E2 reverses the cell physiological effects of unliganded ERα, and promotes gene regulatory effects that are predicted to counter the effects of unliganded ERα. These results reveal that the unliganded form of ERα is not inert, but significantly impacts gene expression and physiology of vascular cells. Furthermore, they indicate that the cardiovascular protective effects of estrogen may be connected to its ability to counteract these effects of unliganded ERα.

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1. Introduction

Until they reach menopause, women have a lower incidence of cardiovascular disease than men. This has been attributed to the protective effects of circulating endogenous estrogen, the major female sex steroid hormone. Indeed, women who undergo ovariectomy, eliminating endogenous estrogen production, have higher risk of CHD and of coronary mortality without estrogen replacement (Parker et al., 2009; Rivera et al., 2009). At a cellular level, 17β estradiol (E2), the active naturally-occurring form of estrogen, reduces the proliferation of vascular smooth muscle cells (VSMCs), whose over-proliferation contributes to vascular occlusive disease in injured or atherosclerotic vessels, thereby contributing to cardiovascular ischemia (Bhalla et al., 1997; Nakamura et al., 2004). In contrast, E2 promotes proliferation of vascular endothelial cells (ECs), an essential aspect of vascular healing that attenuates the progression of occlusive vascular disease (Morales et al., 1995).

In vivo studies have confirmed the protective effects of E2 in animal models of cardiovascular disease (reviewed in Mendelsohn and Karas, 2005). There are two isoforms of the estrogen receptor (ER) transcription factor, ERα and ERβ. Isoform-specific knock out (KO) mouse studies have shown that ERα is required for estrogen-dependent protection from adverse vascular remodeling after injury (including inhibition of smooth muscle cell proliferation and of vascular thickening after injury (Pare et al., 2002) and promotion of re-endothelialization (Brouchet et al., 2001)), and from atherosclerosis (including inhibition of plaque formation and complexity, and reduction of circulating cholesterol (Hodgin et al., 2001)). ERβ is dispensable for the protective effects of estrogen in vascular injury (Brouchet et al., 2001; Karas et al., 1999) but rather contributes to regulation of vascular tone and blood pressure (Zhu et al., 2002). Estrogen can also bind to a structurally-distinct integral membrane protein, GPER, selective activation of which can reduce SMC growth and promote arterial dilation (Haas et al., 2009). GPER knock out also

Abbreviations: CHD, coronary heart disease; CM, conditioned medium; E2, 17-beta-estradiol; EC, endothelial cell; ER, estrogen receptor; GEO, Gene Expression Omnibus; IPA, Ingenuity Pathway Analysis; KO, knock out; TF, transcription factor; TFBC, transcription factor binding site consensus sequence; (V)SMC, (vascular) smooth muscle cell; WT, wild type.

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increases atherosclerosis, although it does not eliminate the effect of endogenous estrogen (comparing gonad intact to ovariectomized female mice) (Meyer et al., 2014). Cell type specific knock out studies indicate that ERα in ECs is required for the effect of E2 on atherosclerosis (Billon-Gales et al., 2009) and on re-endothelialization (Toutain et al., 2009), and that ERα in SMCs (but not in ECs) is required for E2-dependent inhibition of neointima formation after electrical injury (Smirnova et al., 2015).

Estrogen receptors are transcription factors that modulate gene transcription when bound by E2 or other agonists. The mechanisms by which ligand-bound ERs regulate gene expression include direct binding of ER to specific estrogen responsive elements (EREs) in the genome, and also recruitment to chromatin through interaction with other transcription factors, such as SP1, NFκB and AP-1 (Marino et al., 2006). A fraction of ERs are also localized to the plasma membrane, where they initiate rapid signaling to cellular kinases in response to E2, resulting in activation of other transcription factors that can modulate vascular gene expression (Bernelot Moens et al., 2012; Bjornstrom and Sjoberg, 2005; Meyer et al., 2009).

The longstanding model has been that unliganded ERs are largely inert, and that, unless they are activated by specific growth factor signaling, ERs are only functional to regulate transcription when they are bound by E2. However, our prior studies, which showed that ERα is required to mediate the protective effects of E2 after vascular injury, also suggested that, in the absence of E2, injury was worse in wild type (WT) mice than in ERα knockout mice (Pare et al., 2002). In particular, comparing WT and ERαKO mice in the absence of detectable circulating E2, we showed that the presence of ERα increased both SMC proliferation (from a proliferation index of 1.1 ± 0.3 in ERαKO + Vehicle (Veh) to 2.8 ± 0.8 in WT + Veh, p. < 0.05) and medial thickening (from $22.0 \pm 0.8 \times 10^{-3}$ mm^2 in ERαKO + Veh to $27.9 \pm 2.8 \times 10^{-3}$ mm^2, p. < 0.05, (Pare et al., 2002)). This indicated that unliganded ERα might negatively impact vascular function. Furthermore, other recent studies have indicated that unliganded ERα can affect gene regulation and cell proliferation in MCF7 breast cancer cells (Caizzi et al., 2014), and alter neural growth factor responses in PC12 cells (Merot et al., 2009). Taken together, these observations suggested the novel hypothesis that unliganded ERα actively modulates gene regulation and physiology in vascular cells and tissues.

Here, we show that unliganded ERα inhibits the proliferation and migration of ECs, promotes the proliferation of SMCs, and promotes inflammatory effects in both ECs and SMCs in vitro. We also find that unliganded ERα modulates the expression of many genes involved in vascular cell proliferation and movement, both in cultured ECs and in whole vessels. We find that the vascular effects of unliganded ERα are reversed by E2 treatment, and that the predicted physiological effects of gene regulation by unliganded ERα oppose the predicted effects of E2.

2. Material and methods

2.1. Mouse models

All animals were handled in accordance with NIH standards and procedures approved by the Tufts Medical Center Institutional Animal Care and Use Committee. For global gene expression profiling experiments and carotid injury experiments, female ERα KO mice (Dupont et al., 2000) at 3–4 months of age, and their respective wild type female littermates, were ovariectomized. Following one week recovery, the mice from each genotype were randomized to receive 17β-estradiol or placebo pellets (60 day release, 0.25 mg/pellet; Innovative Research of America, Sarasota, Florida). For the expression profiling study, aortas were harvested after one week of E2 or placebo treatment, and RNA isolated as described (O'Lone et al., 2007).

2.2. Ctrl-EC and ERα-EC lines

We recently developed Ctrl-EC and ERα-EC cell lines, as described in (Lu et al., 2016). Briefly, EAhy926 cells (a human umbilical vein endothelial cell hybrid (Edgell et al., 1983), kind gift of C.J. Edgell, University of North Carolina at Chapel Hill), that do not express ERα, were transfected with pCMV3-ERα plasmid ((Karas et al., 1998), "ERα-EC" cells) or control backbone vector pCDNA 3.1 (Invitrogen, "Ctrl-EC" cells) using PolyFect transfection reagent (Qiagen). After 24 h the cells were placed in selective media with 5 μg/ml puromycin (Sigma) for 2–3 weeks. Eight to ten single colonies were selected and maintained in the presence of 2 μg/ml puromycin. ERα expression was confirmed by western blot, as described below. EAhy926 stable cells were grown in 6-well plates to 80% confluence, switched to serum free medium for 24 h, and then treated with 10 nM E2 or ethanol vehicle for 16 h. Total RNA was purified with the RNeasy mini kit (Qiagen) for profiling as described below.

2.3. Luciferase assays & immunoblotting

To test for genomic functions of ERα, stable Eahy926 cell lines (Ctrl-EC and ERα-EC) were grown in phenol red-free DMEM with 10% charcoal-stripped bovine growth serum (sBGS) for 24 h, and transiently transfected with an ERE-luciferase reporter plasmid and β-galactosidase expression plasmid (Karas et al., 1998). 6 h after transfection, cells were switched to serum free medium containing 10 nM 17β-estradiol (E2, Sigma-Aldrich, St. Louis, MO) or ethanol vehicle for 16 h, and normalized luciferase/β-gal values determined, as per (Karas et al., 1998). Each experiment was performed a minimum of three independent times. For western blots, cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the appropriate primary antibody. Antibodies used are: rabbit polyclonal anti-ERα HC20 (Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-AKT, phospho-ERK, total AKT and total ERK (Cell Signaling Technology) and mouse monoclonal anti-GAPDH (Calbiochem). The membranes were then incubated with the appropriate secondary antibody and developed with ECL (Amersham Biosciences).

2.4. Microarray & quantitative RT-PCR

Aortic RNA was processed and hybridized to Affymetrix mouse genome 430 2.0 arrays (3–5 arrays per condition), as described in (O'Lone et al., 2007). EC RNA was processed and hybridized to Illumina HumanHT-12 v4 Expression BeadChip microarrays at the Yale Center for Genome Analysis (YCGA, http://medicine.yale.edu/keck/ycga/microarrays/index.aspx). Differential expression for both types of arrays was determined using Limma (Smyth, 2004). Genes showing at least a 1.3 fold change in expression between ECs or aortas containing or lacking ERα, in the presence of vehicle (ERα-EC + Veh vs. Ctrl-EC + Veh or WT + Veh vs. ERα KO + Veh), or between ECs or aortas containing ERα in the presence or absence of E2 (ERα-EC + E2 vs. ERα-EC + Veh or WT + E2 vs. WT + Veh) and a p. value < 0.01 were considered to be significantly differentially regulated by unliganded ERα or E2, respectively, as per (Lu et al., 2016). The distribution of fold change values for genes that meet these two thresholds, by percentile, was; 10th: 1.33, 25th: 1.38, 50th: 1.51, 75th: 1.75, 90th: 2.28. Selected genes from microarray analysis were further confirmed by qRT-PCR using RNA from new biological samples. RNA was reverse transcribed by using of the QuantiTect reverse transcription kit (Qiagen) and qRT-PCR was carried out using SYBR Green (QIAGEN) and the gene specific primers listed in Suppl. Table B.5. For each gene in ECs, the RNA level was normalized

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