Differential activation by xenoestrogens of ER α and ER β when linked to different response elements

W D Pennie, T C Aldridge and A N Brooks

Endocrinology and Neurobiology Group, ZENECA Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK

Abstract

The discovery of a second estrogen receptor (ER β) has significant implications for our understanding of the molecular basis for the diverse actions of estrogen. Here we report the differential activation by natural and xenobiotic estrogens of ER α and ER β when linked to different response elements. Receptor mediated activation of reporter constructs containing either the estrogen response element (ERE) from the vitellogenin (Vit) gene or from the luteinizing hormone β (LH)

gene were examined in transiently transfected Cos-1 cells. ER β preferentially activated the consensus Vit ERE whereas ER α showed greater activation at the divergent LH ERE. This differential activation was observed for a number of ligands including estradiol, estrone, bisphenol A, octylphenol and diethylstilbestrol. These findings show that the nature of the ERE, as well as the ratio of ER subtypes in a particular cell/tissue, will influence whether particular estrogen responsive genes are activated in the presence of natural or xenobiotic estrogens.

Introduction

A range of environmental chemicals have been shown to mimic the actions of estrogen in a variety of *in vitro* and *in vivo* test systems, and concerns remain over their potential adverse effects on reproductive health in humans and in wildlife species (Gaido *et al.* 1997; Odum *et al.* 1997; Sharpe 1997). The effects of estrogens on a wide variety of organs and tissues have, until recently, been thought to be mediated by a single nuclear hormone receptor, ER α . However, the discovery of a second estrogen receptor ER β has prompted a re-evaluation of the molecular basis for estrogen action (Kuiper *et al.* 1996). Structurally, ER β is highly homologous to ER α in the DNA binding domain (>95% amino-acid identity) but shows only 55% homology in the ligand binding domain. These structural differences lead to different relative binding affinities in ligand binding assays, of a number of estrogenic ligands including xenoestrogens such as methoxychlor and bisphenol A and phytoestrogens such as coumestrol and genestein (Kuiper *et al.* 1997). For both groups of compounds the relative binding affinity was greater for ER β compared with ER α . Furthermore, ER β shows a discrete tissue distribution being the most predominant ER in the prostate, ovary and certain regions of the brain such as the paraventricular nucleus (Kuiper *et al.* 1996; Byers *et al.* 1997; Kuiper *et al.* 1997; Shughrue *et al.* 1997).

Differential estrogen receptor subtype distribution may account for the ability of estrogens to exert tissue specific effects. However, a further way to confer tissue specificity would be provided if estrogen receptor subtypes were able to transactivate different EREs present on estrogen responsive genes in different tissues. The very existence of more than one ER suggests that estrogen responsive genes may contain

EREs which respond differently to either ER β homodimers or ER α /ER β heterodimers (Cowley *et al.* 1997; Petterson *et al.* 1997). ER α and ER β can also mediate gene transcription via an AP1 enhancer site with different effects being observed dependent on ligand and receptor subtype (Paech *et al.* 1997).

The purpose of this study was therefore to determine the extent to which ligand activated estrogen receptor subtypes demonstrate differential activation of diverse estrogen response elements.

Materials and Methods

Cell culture, DNA transfection and luciferase assay

Cos-1 cells were plated (in 24 well dishes) at 1×10^5 cells per well (3% charcoal-stripped calf serum in phenol red free DMEM medium) 48 hours before transfection. DNA was transfected using DOTAP (Boehringer Mannheim) at a total DNA concentration of $1 \mu\text{g}/\text{well}$ (250ng reporter, 500ng ER expression construct, 250ng internal β -gal standard) using $6 \mu\text{l}$ of DOTAP reagent following the manufacturer's protocol. After 5 hours the media was replaced and the cells were allowed to recover overnight before 24 hours of ligand treatment. The cells were harvested in $100 \mu\text{l}$ lysis buffer (Promega). The lysate was then assayed for luciferase and β -galactosidase activity using standard protocols. Transfection efficiency was normalised using the internal β -gal control (pCMVbgal). Data presented are the mean $\pm$ SD for between 3 and 8 individual experiments.

Plasmids

Reporter constructs: Two tandem copies of the estrogen response element from either the vitellogenin gene

(GGTCACAGTGACC) or the luteinizing hormone β gene (GGACAGATGGTGTCC) were cloned upstream of a minimal SV40 promoter driving a luciferase gene (pGL3; Promega) to generate the plasmids p2xVitluc and p2xLH β luc respectively. The plasmids were constructed with the same spacing between the ERE elements and the TATA box (173 bases) and with equivalent spacing between the tandem repeats (AGATCT).

Expression Constructs: The wild type ER α expression construct pSG5Hego (Green *et al.* 1986) was obtained from Dr S. Green (Zeneca Pharmaceuticals). ER β was cloned by RT-PCR from Wistar rat prostate total RNA. Oligonucleotide primers were designed in the basis of the published sequence (5' primer: 5'GCAGAATTCGGACCATGACATTCTACAGTCTTGCT3'; 3' primer: 5'AATTCTAGATTCACTGAGACTGTAGGTTCTG3'). The resulting PCR product was cloned into pcDNA3.1 (Promega) and sequenced, revealing a coding sequence of 1458bp, corresponding to ER β . Our sequence (Accession no. AJ002602) reveals two base pair changes compared with the published sequence for ER β at residues 27 (L to Q) and 105 (P to A).

Results

In this transfection system we observed a striking differential and opposite activation profile for ER α and ER β dependent on the nature of the ERE in the reporter construct. When

stimulated with a range of doses of estradiol 17 β , ER α showed a preference to activate the LH ERE compared with the Vit ERE, whereas the opposite was true for ER β (Fig. 1). These results were consistent for estrone, another naturally occurring estrogen (Fig. 1), and for the xenoestrogens, bisphenol A (BPA) and octylphenol (OP), as well as for the synthetic estrogen, diethylstilbestrol (DES) (Fig. 2). For BPA and OP doses approximately 1000 greater than estradiol were required to produce equivalent inductions of luciferase activity. Cells did not respond to added ligand in the absence of co-transfected estrogen receptor or when transfected with reporter vector alone (no ERE).

Discussion

These results demonstrate for the first time that ER α and ER β can interact differentially with specific estrogen response elements. These data lend further support to the concept that the presence and/or ratio of ER subtypes in a particular cell or tissue will contribute to tissue specific responses to estrogen (Katzellenbogen *et al.* 1997). A number of recent studies have shown that ER β has a unique tissue distribution compared with ER α . For instance, in certain regions of the brain such as the paraventricular nucleus, in situ hybridization analysis has revealed high expression of ER β where there is virtually no expression of ER α (Shughrue *et al.* 1997). ER β is expressed in

Figure 1 Effect of increasing doses of the natural estrogens, estradiol 17 β (E₂), and estrone, on transactivation potential (luciferase activity) of ER α vs ER β on the vitellogenin and luteinizing hormone estrogen response elements. Each histogram represents the mean $\pm$ SD from between 4 and 8 separate experiments.

Figure 2 Effect of increasing doses of the synthetic estrogen, diethylstilbestrol (DES), and the xenoestrogens, bisphenol A (BPA) and octylphenol (OP) on transactivation potential (luciferase activity) of ER α vs ER β on the vitellogenin and luteinizing hormone estrogen response elements. Each histogram represents the mean $\pm$ SD from between 4 and 8 separate experiments.

high abundance relative to ER α in the epithelial cell layer of the secretory alveoli of the prostate (Kuiper *et al.* 1996), in the granulosa cell layer of the ovary (Kuiper *et al.* 1996; Byers *et al.* 1997), and in Sertoli cells, spermatogonia, and spermatocytes of the adult testis (Saunders *et al.* 1998). In addition, ER β exhibits a different ligand binding profile to ER α . For example, ligands such as coumestrol, genistein, methoxychlor and bisphenol A bind with as much as an order of magnitude greater relative binding affinity to ER β compared with ER α (Kuiper *et al.* 1997). This combination of differing tissue distribution, different binding affinities, and our own data demonstrating a preference for different estrogen response elements, may help to explain the many and varied actions of estrogen agonists and antagonists throughout the body (Katzenellenbogen 1996; Katzenellenbogen *et al.* 1997).

The LH ERE used in this study was from the rat LH β gene and has previously been shown to be estrogen receptor regulated by transfection analysis (Shupnik *et al.* 1989) and also to bind ER from uterine or GH3 cell extracts in electrophoretic mobility shift assays (Shupnik *et al.* 1991). In contrast the bovine LH β promoter appears to lack a functional ERE, indicating that species differences exist in the extent to which the LH gene is directly regulated by estrogen (Keri *et al.* 1994). In our studies we have observed that cells do not respond to added ligand in the absence of co-transfected estrogen receptor or when transfected with reporter vector

alone (no ERE). Thus, our observed inductions are clearly operating through the exogenously added estrogen receptors and are dependent upon the cloned estrogen response elements. These data therefore demonstrate that the LH ERE is operating as a functional response element in these assays. The magnitude of the fold inductions we observed in our transient transfection system were slightly less than those found by Petterson *et al.* 1997. However, this is likely to reflect the differing cell types (293 vs Cos-1 cells) and conditions employed in these assays. We have used Cos-1 cells for their ease of transfection and because they contain no endogenous estrogen receptor. It is possible that as our reporter constructs are being driven by a minimal SV-40 promoter, they may have a higher basal activity in Cos-1 cells than in other cell types (due to the presence of SV40LT in these cells), which could lead to a blunting of response.

The finding that ER α and ER β differentially activate specific estrogen response elements is intriguing given the close homology between the DNA binding domains of these receptors (approx. 95%). The specificity reported here may be attributable to the two amino-acid residue differences between the DNA binding domains of these two receptors. In addition it is possible that receptor binding to different ERE sequences may give rise to different conformational changes in the receptor domains which interact with other transcription factors or accessory proteins (Lefstin *et al.* 1994).

Alternatively, the ability of the DNA binding domain of individual receptor subtypes to interact with an ERE, may be influenced by sequences outside the DNA binding domain, as has been suggested for the glucocorticoid receptor (Xu *et al.* 1996). Another way in which estrogen can mediate gene transcription is indirectly, by interacting with the transcription factors Fos and Jun at an AP1 element. A recent report describes differential activation by ER α and ER β via this mechanism (Paech *et al.* 1997). Using an AP1 element reporter system, estradiol caused transcriptional activation through ER α in a ligand independent manner. In contrast ER β caused transcriptional activation when stimulated with estrogen antagonists such as raloxifene, tamoxifen and ICI 164384, and transcriptional inhibition when stimulated by estradiol. Our own studies have explored a different mechanism involving a direct action of estrogen receptors at diverse estrogen response elements. In this system the differential activation we observed was ligand independent with DES, estradiol, estrone, bisphenol A and octylphenol all having the same effects. The dose of bisphenol A and octylphenol required to cause transcriptional activation was several fold greater than for estradiol. These data are consistent with their reported weak estrogenic effects (Shelby *et al.* 1996; Steinmetz *et al.* 1997) and provide evidence that xenoestrogens, like natural estrogens, are able to cause transcriptional activation through both forms of estrogen receptor.

In conclusion, our data demonstrate that the nature of the ERE, as well as the ratio of ER subtypes in a particular cell/tissue may influence whether particular estrogen responsive genes are activated in the presence of natural or xenobiotic estrogen. They further demonstrate the necessity to consider both the type of estrogen receptor and the ERE when designing mammalian cell transactivation reporter assays for evaluating the estrogenic potential of environmental chemicals.

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