Molecular and Cellular Endocrinology 276 (2007) 45–54

www.elsevier.com/locate/mce

A novel molecular assay to discriminate transcriptional effects caused by xenoestrogens

Mohammad Kazem Koohi [a,1], Norbert Walther [a,2], Richard Ivell [b,*]

[a] *Institute for Hormone and Fertility Research, University of Hamburg, 20246 Hamburg, Germany*
[b] *School of Molecular and Biomedical Science, University of Adelaide, Adelaide SA5005, Australia*

Received 15 March 2007; received in revised form 28 June 2007; accepted 29 June 2007

Abstract

A phenotypic definition of the term estrogen has become increasingly problematic due to the multiple modes of estrogen action which can now be defined by differing nuclear and membrane receptors for the classic ligand, 17β-estradiol, and by the multiple signalling pathways that are consequently addressed. This has led to the term xenoestrogen being largely determined by whatever assay system is used for its definition. Here we describe a novel and simple matrix for a transfection system using MBA-MD231 and MCF-7 breast cancer cells as hosts. This matrix is able to vary the type of nuclear estrogen receptor used, and by varying the promoter-reporter construct between one using a classic estrogen response element (ERE) enhancer, and one using an enhancer element derived from the bovine oxytocin gene promoter binding an orphan nuclear receptor, direct classical effects can be neatly discriminated from non-classical and non-genomic actions of test substances. This assay matrix has been used to examine a selection of phytoestrogens and xenobiotics, thereby providing new information on the mechanism of action of some of these substances in breast cancer cells.

Keywords: Xenoestrogen; Estrogen receptor; Oxytocin gene; Phytoestrogen

1. Introduction

There is considerable confusion in the literature caused by the diverse usages of the term "estrogen". It has been used in its biological sense to denote a substance able to induce feminization in male fish (Matthiessen and Sumter, 1998), reinforced by the observation that a principal xenoestrogen in natural waters was identified as the estrogen receptor (ER) agonist ethinyl estradiol (Matthiessen and Sumter, 1998). The application of the E-screen defined an estrogen as a substance able to induce proliferation of cultured breast cancer cells (Baker, 2001). The development of screening tests based on reporter gene assays provide a mechanistic definition whereby an estrogen is a substance able to induce the transactivation of an ERα-dependent reporter gene, acting directly via a classical estrogen responsive element (ERE) in the promoter of that gene (Baker, 2001). Molecular research has recently highlighted a very great degree of complexity in the way substances can exert estrogenic effects. Application of microarrays to assess genes up-regulated by the *in vivo* application of the endogenous estrogen 17β-estradiol in mammals indicates that the classic mechanism of action defined by direct ERα activation of ERE-containing genes probably accounts for only a small proportion of all genes induced by estrogens (Shioda et al., 2006). Firstly, it is now known that there are multiple estrogen receptors, some of which act as ligand-activated nuclear transcription factors (ERα and ERβ), some of which are G-protein coupled receptors at the cell membrane (Filardo and Thomas, 2005). Secondly, it is now recognized that, for many of the estrogenic effects involving the nuclear estrogen receptors ERα and ERβ, these may not require a direct interaction between the hormone receptor and the classic ERE in the promoter of a gene (Glidewell-Kenney et al., 2005; Song et al., 2005). Rather, the ligand-activated receptor may interact with other proteins causing activation of intracellular kinase cascades (Song et al., 2005; Lee et al., 2005), or of other transcription factors with quite different DNA-binding properties (Wang et al., 1999). It is this possibility for different mechanisms of estrogen action that is believed to explain the tissue-specificity of the compounds,

* Corresponding author.
E-mail address: richard.ivell@adelaide.edu.au (R. Ivell).
[1] Present address: Faculty of Veterinary Medicine, University of Tehran, Iran.
[2] Present address: School of Life Science Hamburg, University Hospital Eppendorf, Hamburg, Germany.

0303-7207/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2007.06.008

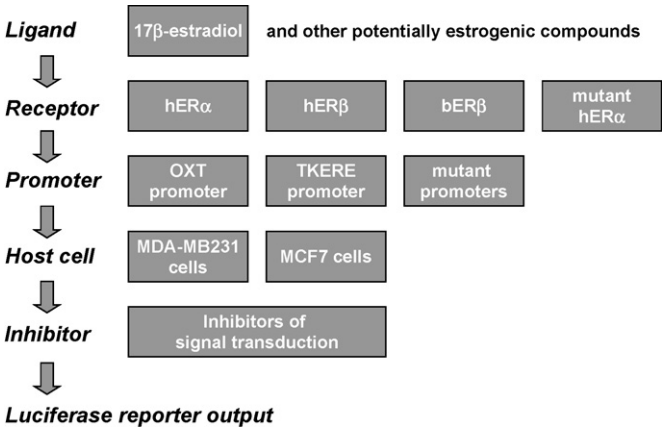

Fig. 1. Matrix to show the combinations of factors, compounds, promoters and cells that are exchangeable used in this novel assay system.

known as SERMs (selective estrogen receptor modulators) (Safe and Papineni, 2006). The conformation of an estrogen receptor induced by binding of a SERM may act agonistically in one molecular context, and antagonistically in another.

We have recently developed a simple *in vitro* assay system (Fig. 1), which is able to discriminate clearly between different SERM effects, and thus is able to highlight quite different estrogenic actions from those defined by the more restrictive estrogen-dependent molecular assays (Koohi et al., 2005). This new assay exploits the fact that an important promoter element from the bovine oxytocin gene, which *in vivo* is known to interact with the monomeric orphan nuclear receptor, steroidogenic factor 1, can also respond to ligand-activated estrogen receptors, although these cannot bind to it (Stedronsky et al., 2002; Koohi et al., 2005). Furthermore, this action of estrogen can be largely inhibited by addition of the specific MAP-kinase inhibitor PD98059 (Koohi et al., 2005). By varying the components of the system (different estrogen receptors, different promoter elements, different combinations of estrogen agonists and antagonists, different pharmacological inhibitors), we are able to discriminate very clearly agonist and antagonist actions, for example, of the SERMs, tamoxifen and raloxifen (Koohi et al., 2005), in particular showing agonist activity, which would not have been detected in more conventional ERE-dependent assays.

In the present investigation we have further developed this assay system to explore the estrogenicity of some common environmental xenobiotics and phytoestrogens, as well of some metal ions common in contaminated waters.

2. Materials and methods

2.1. Transfection assays

The majority of methods and materials are described in detail in the previous publication (Koohi et al., 2005). In addition to the MDA-MB231 cells used exclusively in the earlier study, also a relatively early passage of MCF-7 cells was used, exactly as previously described (Stedronsky et al., 2002). Both types of cell were seeded at a density of 10^5 cells per well into 12-well plates, and on the following day transfected with a total of 3 μg of plasmid DNA using the calcium phosphate coprecipitation procedure (Profection Mammalian Transfection System, Promega, Mannheim, Germany). The transfected plasmid DNA comprised 1 μg of promoter-luciferase plasmid, 1.5 μg of the relevant ER expression vector (see below), and 0.5 μg of a LacZ control vector driven from the CMV early promoter. Following transfection, cells were incubated for 16 h, then medium was changed, and cells were stimulated or not with various effectors in 2.5 ml fresh culture medium per well. After a further 24 h incubation, cells were washed twice in PBS and then lysed in 1× Passive Lysis Buffer (Promega) before determination of luciferase and β-galactosidase activities (Koohi et al., 2005). After correction for transfection efficiency, results are expressed as relative light units (means + S.D. for triplicate wells). All experiments were repeated at least twice with identical results. Statistical significance for the differences in promoter activities was assessed by one-way ANOVA followed by the Newman–Keuls test, or unpaired *t*-tests with Welch's correction using the GraphPad Prism 3.0 software package (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

2.2. DNA constructs

All promoter-reporter constructs are as previously described (Koohi et al., 2005). Either the bovine oxytocin promoter (−183 to +17; Ruppert et al., 1984) was used (OXT), inserted into the pGL3-Basic vector (Promega), or the thymidine kinase promoter controlled by a single vitellogenin ERE (TKERE) was used to drive a similar luciferase reporter construct (Stedronsky et al., 2002). As negative and positive controls, respectively, we used the pGL3-Basic plasmid, containing neither promoter nor transcriptional enhancer sequences, and the pGL3-Control vector, expressing luciferase under the control of the SV40 promoter and enhancer (both from Promega). ERα was generated from an expression construct comprising the human ERα cDNA controlled by a CMV viral promoter (Koohi et al., 2005). A version of ERα wherein the DNA-binding domain (DBD) was mutated so that the receptor is no longer able to interact directly with an ERE was generated as in Koohi et al. (2005). A human ERβ (hERβ) expression vector was obtained as a generous gift from Dr. Katrin Stedronsky (Institute for Hormone and Fertility Research, Hamburg, Germany) and the bovine ERβ (bERβ) expression construct was prepared as in Walther et al. (1999) also driven from a CMV promoter.

2.3. Chemicals and xenobiotics

17β-Estradiol (E2; 1 nM = 0.272 ng/ml), 4OH-tamoxifen (1 nM = 0.387 ng/ml), raloxifen (1 nM = 0.510 ng/ml), and the antiestrogen ICI182,780 (1 nM = 0.609 ng/ml) were all obtained from Sigma–Aldrich (Deisenhofen, Germany). Naringenin (1 nM = 0.272 ng/ml), 6-(1,1-dimethylallyl) naringenin (6DMA-naringenin; 1 nM = 0.340 ng/ml), and 8-prenylnaringenin (8p-naringenin; 1 nM = 0.340 ng/ml) were a generous gift from Professor Gunter Vollmer (University of Dresden, Germany). β-HCH (β-hexachlorcyclohexane; 1 nM = 0.291 ng/ml) was purchased from Supelco (Bellefonte, PA), *o′p′*DDT (1.1.1.-trichloro-2-(*o*-chlorophenyl)-2-*p*-chloriphenyl)ethan; 1 ng = 0.352 ng/ml) from Chem Service (West Chester, PA), *p′p′*DDE (2-2-bis (4/chlorophenyl)-1-1-dichloroethyl; 1 ng = 0.318 ng/ml), methoxychlor (1,1,1-trichloro-2-2-bis-(*p*-methoxyphenyl)ethane-2-2-bis(4-methoxyphenyl)1-1-1-trichloroethan; 1 ng = 0.347 ng/ml), chlordane (1 nM = 0.410 ng/ml), 2OH-biphenyl (1 nM = 0.170 ng/ml), genistein (1 nM = 0.270 ng/ml), toxaphen (1 nM = 0.414 ng/ml), and resveratrol (3,4,5-trihydroxy-*trans*-stilbene,5-(1*E*)-2-(4-hydroxyphenyl)-1-3-benzenediol; 1 nM = 0.228 ng/ml) were all from Sigma–Aldrich. $ZnCl_2$ and $CoCl_2$ were from Fluka (Neu-Ulm, Germany), LiCl and $MgCl_2$ from Merck (Darmstadt, Germany), and $CuCl_2$, $HgCl_2$ and $CdCl_2$ from Sigma–Aldrich.

2.4. Toxicity testing

All compounds used were additionally tested for any cytotoxic effects at the concentrations used, which could influence the results obtained. Firstly, cells were checked for integrity following a 24 h incubation with the test substance by staining washed cells for 3 h with 2% neutral red, and after further washing in PBS, photometrically measuring the absorbed dye at 540 nm. Secondly, following exposure to the test substances, cells were replated into T75 flasks, and subjected to 7 days of culture, after which all colonies in the flasks were stained with 10% Giemsa (Merck) and cells counted.