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Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption☆

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Abstract

A wide variety of environmental contaminants have been shown to exert estrogenic actions in wildlife and laboratory animals through binding to nuclear estrogen receptors (ERs) and subsequent transcription of estrogen responsive genes. We show here that several of these environmental estrogens also bind to the novel seven-transmembrane estrogen receptor, GPR30, to activate alternative estrogen signaling pathways in an ER-negative cell line (HEK293) stably transfected with the receptor. Genestein was the most effective competitor for the receptor (IC_{50} 133 nM), with a relative binding affinity (RBA) 13% that of estradiol-17β (E2). Bisphenol A, zearalonone, and nonylphenol also had relatively high binding affinities for GPR30 with RBAs of 2–3%. Kepone, *p*,*p*′-DDT, 2,2′,5′,-PCB-4-OH and *o*,*p*′-DDE had lower affinities with RBAs of 0.25–1.3%, whereas *o*,*p*′-DDT, *p*,*p*′-DDE, methoxychlor and atrazine caused less than 50% displacement of [^{3}H]-E2 at concentrations up to 10 μM. Overall, the binding affinities of these compounds for GPR30 are broadly similar to their affinities to the ERs. Environmental estrogens with relatively high binding affinities for GPR30 (genestein, bisphenol A, nonylphenol and Kepone) also displayed estrogen agonist activities in an *in vitro* assay of membrane-bound adenylyl cyclase activity, a GPR30-dependent signaling pathway activated by estrogens. The results indicate that nontraditional estrogen actions mediated through GPR30 are potentially susceptible to disruption by a variety of environmental estrogens.

Keywords: GPR30; Environmental estrogens; Endocrine disruption; Pesticides; Phytoestrogens; Nongenomic steroid action

1. Introduction

The undesirable estrogenic actions of certain anthropogenic chemicals such as bisphenol A and the pesticide dichlorodiphenyl-trichloro-ethane (DDT) have been known since the mid twentieth century [1,2]. Early on it was demonstrated that the estrogenicity of *ortho*, *para* derivatives of DDT as well as several polychlorinated biphenyl (PCB) metabolites was estrogen receptor (ER)-mediated [3]. However, research on environmental estrogens has increased dramatically over the past 15 years because of a heightened awareness and concern over their endocrine disrupting effects, resulting in alterations in reproduction and development in fish, wildlife and humans [4]. The production of the estrogen-induced yolk precursor protein, vitellogenin, in freshwater and marine fish, and feminization of male birds, alligators and fish have been observed after environmental exposure to estrogenic chemicals such as *o*,*p*′-DDT and Kepone, and to effluents from water treatment plants containing nonylphenols and ethynylestradiol [5,6]. Environmental estrogens have also been implicated in breast and endometrial cancers in humans [7]. The number of environmental contaminants shown to be estrogenic has increased dramatically over the last decade. The majority of estrogenic compounds including *ortho*, *para* derivatives of DDT, bisphenol A, nonylphenol, methoxychlor metabolites, PCBs and their hydroxylated metabolites, Kepone and phytoestrogens such as genestein and zearalonone are thought to exert their estrogenic effects primarily by binding to the ER [3,8,9]. However, other mechanisms such as alterations of growth factors or

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cross-talk through binding to the aryl hydrocarbon receptor and interference with the transcriptional activity of the ER [10–12] have also been implicated in chemical disruption of estrogen actions.

There is now extensive evidence in a broad range of animal and cell models that steroid hormones can also exert rapid, nongenomic (i.e. non-classical) actions initiated at the cell surface by binding to specific steroid membrane receptors [13–15]. Ligand binding to these steroid membrane receptors causes rapid activation of second messenger pathways, such as increases in intracellular free calcium or cAMP, resulting in a cellular response [16,17]. The importance of this alternative mechanism of steroid action is becoming more widely recognized as recent studies implicate steroid membrane receptor-mediated mechanisms in a growing number of physiological functions. The results of recent studies suggest that nongenomic steroid actions, like genomic ones, are susceptible to interference by environmental estrogens [18]. The environmental estrogens that bind to the ER are also effective competitors for [^{3}H]E2 binding to estrogen membrane receptors [19] and have been shown to exert agonist effects on nongenomic estrogenic actions in several fish and tetrapod models [19–22]. However, our current lack of precise information on the identities of many steroid membrane receptors has prevented the development of conceptual models of their molecular interactions with environmental estrogens.

Recently, a novel seven-transmembrane receptor, GPR30, that mediates nongenomic estrogen actions to up-regulate adenylyl cyclase and MAPkinase activities [23] through activation of a stimulatory G protein was identified in ER-negative SKBR3 breast cancer cells [24]. Recombinant GPR30 protein, produced in ER-negative HEK231 cells, shows all the steroid binding and signaling characteristics of a functional estrogen membrane receptor [24]. GPR30 is the first estrogen membrane receptor, structurally unrelated to the nuclear ERs, whose molecular structure is known. Therefore, in the present study the ability of environmental estrogens to bind to recombinant GPR30 and activate GPR30-mediated second messengers was investigated.

2. Materials and methods

2.1. Chemicals

The DDT derivatives, *o*,*p*′-DDT, *p*,*p*′-DDT and *p*,*p*′-DDE, and atrazine were purchased from Chem Service (Westchester, PA). The hydroxylated polychlorinated biphenyl, 2,2′,5′-trichloro-4-biphenylol (2,2′,5,-PCB-4-OH) was purchased from Ultra Scientific (North Kingston, RI). The chlorinated pesticides Kepone and methoxychlor were obtained from the National Institute of Environmental Health Sciences Repository (NIH, Bethesda, MD). The nonionic surfactant 4-nonylphenol (nonylphenol) was obtained from the Huntsman Corporation (Port Neches, TX). The phytoestrogen genestein, bisphenol A and estradiol-17β were purchased from Sigma Chemical Company (St. Louis, MO).

2.1.1. Culture of HEK231 cells stably transfected with GPR30

Human HEK293 cells were stably transfected with GPR30 as described previously [24] and cells expressing the GPR30 construct were selectively maintained with 500 μg/ml geneticin. Cells were cultured in DMEM/Ham's F-12 medium without phenol red supplemented with 5% fetal bovine serum (FBS) and 100 μg/ml of gentamicin in 150 mm diameter plates. The medium was replaced every 1–2 days and the cells reached 80% confluence after 3 days culture, at which time the medium was replaced with medium lacking FBS and the cells were cultured for another day before use in the experiments.

2.1.2. Estrogen receptor competition studies

General procedures used in our laboratory for the preparation of plasma membrane fractions and assay of steroid membrane receptors were followed with few modifications for measurement of [^{3}H]E2 binding to plasma membranes of HEK293 cells transfected with GPR30 [24]. Specific E2 binding was calculated from binding of 4 nM [2,4,6,7-^{3}H]E2 ([^{3}H]E2, ∼89 Ci/mmol) alone (total binding) and binding in the presence of 100-fold excess (400 nM) nonradioactive E2 (nonspecific binding). The competitive binding assay tubes contained 4 nM [^{3}H]E2 and the steroid competitors (concentration range: 1 nM to 10 μM; dissolved in 1 μl ethanol). After a 30-min incubation at 4 °C with the membrane fractions, the reaction was stopped by filtration (Whatman GF/B filters), the filters were washed and bound radioactivity measured by scintillation counting. The displacement of [^{3}H]E2 binding by the steroid competitors was expressed as a percentage of the maximum specific binding of E2.

2.1.3. Adenylyl cyclase activity

Adenylyl cyclase activity was determined by measuring the production of cAMP by plasma membranes of transfected cells after treatment with E2 (100 nM) or other test compounds (200 nM) for 20 min at 25 °C as described previously [24]. cAMP concentrations were subsequently measured in cytosolic fractions using a EIA kit following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

3. Results

Several of the environmental estrogens, genestein, bisphenol A, nonylphenol, Kepone and 2,2′,5′,-PCB-4-OH, displaced at least 50% of the [^{3}H]E2 binding to plasma membranes of cells transfected with GPR30, indicating they have the potential to interfere with estrogen signaling through this membrane receptor as agonists or antagonists (Fig. 1). The