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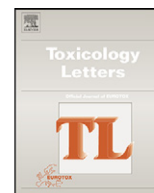

Development of a competitive binding assay system with recombinant estrogen receptors from multiple species☆

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ABSTRACT

In the current study, we developed a new system using full-length recombinant baculovirus-expressed estrogen receptors which allows for direct comparison of binding across species. Estrogen receptors representing five vertebrate classes were compared: human estrogen receptor alpha (hERα), quail estrogen receptor alpha (qERα), alligator estrogen receptor alpha (aERα), salamander estrogen receptor alpha (sERα), and fathead minnow estrogen receptor alpha (fhERα). Saturation binding analyses indicated 17β-estradiol (E2) dissociation constants (Kd) were 0.22 ± 0.02 nM for hERα, 0.28 ± 0.04 nM for sERα, 0.44 ± 0.04 nM for aERα, 0.58 ± 0.10 nM for qERα, and 0.58 ± 0.05 nM for fhERα. Binding specificity to each of the receptors was evaluated using E2, dihydrotestosterone (DHT), corticosterone (C), and ethinylestradiol (EE). E2 and EE were strong binders in all species with IC50's ranging from 0.65 nM with hERα to 1.01 nM with sERα for E2 and from 0.68 nM with sERα to 1.20 nM with qERα for EE. DHT was a weak binder with IC50's ranging from 3.3 μM with hERα to 39 μM with fhERα, and C did not bind any of the receptors at concentrations up to 100 μM. This system provides a convenient in vitro approach for directly comparing chemical binding to estrogen receptors across multiple species without the need to sacrifice animals.

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1. Introduction

In a report prepared for the US EPA, the National Research Council recommended shifting the focus of toxicity testing from chemical-driven animal studies to pathway disruption analyses that incorporate high throughput in vitro techniques and decrease the use of animals (NRC, 2007). Along these lines, the proposed tiered approach for screening and testing of potential endocrine disrupting chemicals (EDCs) incorporates in vitro assays using exclusively mammalian receptors to assess chemical binding to estrogen and androgen receptors. Few studies, however, assess whether compounds display consistent chemical binding profiles across vertebrate classes. This is in part due to the difficulty in acquiring multiple full-length receptors and incorporating them into a consistent binding platform that would allow for a direct comparison.

Existing estrogen receptor (ER) binding assays that compare chemical binding across species have used both receptor constructs and receptors prepared from tissues. For example, Matthews et al. developed a recombinant ER binding protocol which used fusion proteins from multiple species that included the hinge (D domain), ligand binding (E domain) and variable (F domain) regions of the receptor (Matthews et al., 2000). Other scientists have used ER preparations from cells or tissues to compare binding in two different fish species or between fish and humans (Denny et al., 2005; Olsen et al., 2005). Both approaches differ from our current system which uses full-length recombinant estrogen receptors alpha of representative species from five vertebrate classes (boney fish, amphibian, reptile, bird, and mammal).

In the current study, we describe a simplified and higher throughput estrogen receptor binding assay. In addition to human estrogen receptor alpha (hERα), we also generated vectors expressing estrogen receptors alpha from fathead minnow (*Pimephales promelas;* fhERα) Japanese quail (*Coturnix japonica*; qERα), Japanese giant salamander (*Andrias japonicus*; sERα), and American alligator (Alligator mississippiensis; aERα). In order to confirm that these receptors were functional and performed as expected, we

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assessed their performance in saturation binding and competitive binding assays with four steroids having a range of affinities for ER.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E2; CAS 50-28-2, purity 98%), 17a-ethynylestradiol (EE; CAS 57-63-6, purity ≥98%), corticosterone (C; CAS 50-22-6, purity ≥92%), and dihydrotestosterone (DHT; CAS 521-18-6, purity ≥97.5) were purchased from Sigma-Aldrich (St. Louis, MO). Radiolabeled [2,4,6,7,16,17-^{3}H(N)] 17β-estradiol was purchased from PerkinElmer (Boston, MA). The purities of all chemicals used in this study were determined by their manufacturers.

2.2. Baculovirus constructs

hERα in a baculovirus expression vector (AcVHER) was a generous gift from C. Klinge (Klinge, 1999). The isolation of fhERα from a cDNA library and subcloning into the expression plasmid, pFERa-g, was described previously (Wilson et al., 2004). The fhERα gene was originally cloned into the pBSSK minus plasmid and isolated from that plasmid by PCR (Wilson et al., 2004). The isolated gene was digested with Bam HI and Eco RI, and the gel purified fragment was subcloned into transplasmid pVL1393, yielding the new plasmid pVL1393FER. Linear AcV EPA DNA and pVL1393FER were co-transfected into Sf21 insect cells to yield recombinant baculovirus AcVFER (Hartig and Cardon, 1992).

Estrogen receptor alpha sequences from the American alligator (*Alligator mississippiensis*; GenBank accession no. AB115909 (Katsu et al., 2004)), the Japanese quail (*Coturnix japonica*; Genbank accession no. AF442965), and the Japanese giant salamander (*Andrias japonicus*; Genbank accession no. AB252211 (Katsu et al., 2006)) were publicly available. Full-length coding sequences capable of generating proteins for aERα, qERα, and sERα were synthesized by GenScript Corporation (Piscatway, NJ, USA). GenScript proprietary algorithms were utilized to optimize the sequences for synthesis and to remove problematic internal restriction sites while adding a 5′ and a 3′ Eco R1 site. Translation of the sequences predicted a protein identical to that predicted from the original published sequences (available upon request). The ER genes were each initially cloned into plasmid pUC57 and then digested, gel purified, and subcloned into the Eco R1 site of transplasmid pVL1393 (Invitrogen, Grand Island, NY, USA). pVL1393 containing the target ER was transfected into Sf21 insect cells along with AcV EPA DNA as described above. Baculovirus clones AcValER (alligator), AcVqER (quail), and AcVsER (salamander) were selected based on PIB minus phenotype and isolated. These baculovirus constructs express the ER gene under control of the polyhedrin promoter.

2.3. Receptor production

General insect cell culture and baculovirus manipulation techniques used to express proteins are described in detail elsewhere (O'Reilly et al., 1992; Summers and Smith, 1987). Receptors for assays were produced in 50 ml suspension cultures. Sf21 cells were infected at a multiplicity of infection of one, incubated 72 h at a cell density of 1×10^6/ml, and centrifuged at $700 \times g$ for 10 min. The resulting pellet was suspended in 50 ml of high salt TEDG buffer (400 mM KCl, 10 mM Tris, 10% glycerol, 1 mM sodium molybdate, 1.5 mM EDTA, 1 mM PMSF, and 1 mM DTT) (Wilson et al., 2002), freeze thawed on ice 3 times, clarified by centrifugation ($12,000 \times g$ at 4 °C for 30 min.), and frozen at −80 °C until used.

2.4. Receptor concentration

For each new batch of receptor produced, range-finding experiments were conducted to determine the optimum receptor concentration for use in binding assays. Total and non-specific binding were measured using serially diluted insect cell lysate corresponding to decreasing receptor concentrations. The optimum receptor concentration was judged to be one that resulted in an ample dynamic range, but was not so high as to bring about an unacceptable level (>10%) of radioligand bound (i.e. a receptor concentration that results in approximately 5 to 10% specific binding out of total radioactivity added).

Insect cell lysate containing receptor was diluted in TEDG buffer plus 10 mg/ml BSA. Total binding wells contained [^{3}H] E2, while non-specific binding wells contained [^{3}H] E2 with 100-fold molar excess of unlabeled E2.

2.5. Saturation binding

To measure saturation binding, diluted receptor preparations were incubated with increasing concentrations of [^{3}H] E2 (0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, or 2 nM) both alone and with 100-fold molar excess unlabeled E2. Two additional higher concentrations of [^{3}H] E2 (3 and 4.5 nM) were added with some receptors (i.e. sERα, aERα, and fhERα) to assure saturation of receptor binding.

2.6. Competitive binding

Binding assays were performed in 96 well round bottomed plates. All components were kept on ice or at 4 °C throughout binding experiments. The 4 °C temperature of the assay is consistent with other common cell-free binding assays and is necessary to inactivate ubiquitous proteases which could degrade the receptor proteins. Each experiment included 3 replicates of total binding wells, non-specific binding wells, eight concentrations of E2, and ten concentrations each of EE, DHT, and C. A similar protocol for androgen receptor binding is discussed in detail elsewhere (Hartig et al., 2008). Briefly, either test chemical, buffer (total binding), or unlabeled E2 (non-specific binding) were added with 1 nM/well ^{3}H E2, and the receptor preparation was incubated overnight (18–24 h) at 4 °C. Following incubation, free ligand was separated from receptor bound ligand by adding 50 μl of 5% dextran-coated charcoal in TEDG with 10 mg/ml BSA buffer to each well, incubating the plate at 4 °C with 10 min of gentle shaking, and then centrifuging the plate at $1000 \times g$ for 5 min. A 50 μl sample of the supernatant from each well was transferred to a scintillation vial and radioactivity was measured on a Beckman LS 5000TD (Irvine, CA) scintillation counter. Counts were multiplied by 3 to adjust numbers from the aliquot counted to reflect the total volume (150 μl) of the reaction. Competitive binding assays were repeated three times with at least triplicate wells per concentration per run.

2.7. Statistical analysis

Saturation binding data were analyzed using the Kell Ligand software version 6.0.12 (Biosoft, Cambridge, UK), which provides Kd and Bmax estimates through non-linear iterative curve-fitting procedures. Competitive binding data were graphed in Prism (GraphPad Prizm version 5, San Diego, CA) and fit with the one site competition function to calculate IC50 values. When chemicals did demonstrate receptor binding but did not completely displace E2 (i.e. DHT), the bottom of the binding curve was constrained to 0 in order to estimate an IC50 for comparison purposes. Chemicals that displaced less than 50% E2 at the highest soluble concentration were considered non-binders (NB), whereas chemicals that displaced greater than 50%, but less than 80% were considered equivocal binders. To detect significant differences among Kd values, Bmax values and steroid IC50 s among receptors, we preformed Holm-Sidak pairwise multiple comparison procedures for one-way analysis of variance using SigmaStat (Systat Software Inc., San Jose, CA).

3. Results

3.1. Receptor concentration

We identified the concentration of each receptor that resulted in specific binding of 5%–10% of total radioactivity added (see example in Fig. 1). The dilution of concentrated insect cell lysate used in the binding assays varied between batches

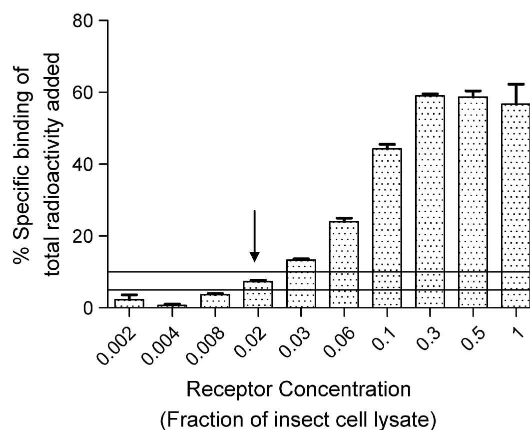

Fig. 1. Example of receptor concentration range finding with sERα. Dilutions of insect cell lysate containing baculovirus expressing sERα were incubated with radiolabeled [^{3}H] 17β-estradiol (E2) alone (total binding) and with 100-fold excess unlabeled E2. Specific binding (total minus non-specific) was then divided by total E2 added to calculate the percent of total bound specifically to the receptor. Each data point represents the mean value ± standard error of three replicates from one assay. The horizontal lines indicate the 5% and 10% levels of total radioactivity added. The arrow indicates the dilution of receptor selected for use in subsequent binding assays.

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