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A stable fish reporter cell line to study estrogen receptor transactivation by environmental (xeno)estrogens

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ABSTRACT

Cross-species differences between human and fish estrogen receptor (ER) binding by environmental chemicals have been reported. To study ER transactivation in a fish cellular context, we stably co-transfected the PLHC-1 fish hepatoma cell line with a rainbow trout estrogen receptor (rtER) and the luciferase reporter gene driven by an estrogen response element (ERE). This new cell model, called PELN-rtER (for *P*LHC-1-*E*RE-*L*uciferase-*N*eomycin), responded to 17β-estradiol (E2) in a both concentration- and temperature-dependent manner, as well as to environmental ER ligands from different chemical classes: natural and synthetic estrogens, zearalenone metabolites, genistein, alkyphenoles and benzophenone derivatives. The comparison with other *in vitro* models, i.e. human reporter cell lines (HELN-rtER, MELN) and vitellogenin induction in primary cultures of rainbow trout hepatocytes, showed an overall higher sensitivity of the human cells for a majority of ligands, except for benzophenone derivatives which were active at similar or lower concentrations in fish cells, suggesting species-specificity for these substances. Correlation analyses suggest that the fish cell line is closer to the trout hepatocyte than to the human cell context, and could serve as a relevant mechanistic tool to study ER activation in fish hepatic cellular context.

1. Introduction

The widespread presence of endocrine disrupter compounds (EDCs) in the aquatic environment has become a very important issue of environmental concern over the past few decades, as these natural or man-made chemicals may cause adverse effects on wildlife (Sumpter, 2005). Given the complexity of the endocrine system as well as the diversity of chemicals and their modes of action, tiered approaches have been proposed for the screening (Tier 1) and testing (Tier 2) of EDCs (reviewed by Hotchkiss et al. (2008)). Tier 1 includes both *in vitro* and short term *in vivo* assays. In this context, the evaluation of non mammalian *in vitro* screening assays has been clearly identified as an important need to be addressed in EDC testing strategies (Hotchkiss et al., 2008). However, compared to mammalian species, fewer non mammalian *in vitro* screening assays have been developed. The lack of species-specific screening assay may represent an important gap in risk assessment of EDCs for aquatic organisms, and for fish in particular, since cross-species differences have been identified with regard to the molecular mode of hormone action (i.e. receptor binding affinities) or xenobiotic metabolism (Matthews et al., 2000; Wilson et al., 2007; Hotchkiss et al., 2008).

One important mechanism in EDC action is mediated by the modulation of estrogen receptor (ER) activation. Different assays exist to assess estrogenic activity of chemicals in fish. Among them, the most widely used is based on vitellogenin (VTG) induction in isolated fish hepatocytes (e.g. Pelissero et al., 1993; Smeets et al., 1999). Such *in vitro* assay is toxicologically relevant because it measures natural gene response in cultured cells derived from a main target organ of EDC (i.e. liver) and because it retains metabolic properties close to the *in vivo* situation. However, it has also some limitations for screening purpose since it is relatively time-consuming and may be the subject of inter-assay variability (reviewed by Navas and Segner (2006)). On the other hand, the use of receptor mediated expression of stable reporter gene system using established cell lines serves as rapid, reproducible and specific assay. However to our knowledge, only few stable reporter gene assays using fish cell lines have been described (Ackermann et al., 2002), and none in hepatic cell context.

In this study, we describe the development of a new stable reporter gene assay for the assessment of ER activation by chemicals in fish cellular context, by using the PLHC-1 hepatoma fish cell line (Ryan and Hightower, 1994). In this model, kinetics of luciferase transactivation by estradiol as function of exposure duration and temperature were determined, as well as its activation by various ER ligands representative of different chemical classes. Finally, the comparison of this new *in vitro* model with other well established

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assays for estrogenicity assessment, namely VTG induction in isolated rainbow trout hepatocytes and human reporter cell lines derived from HeLa (HELN-rtER) and MCF-7 (MELN) cells, highlighted some response specificity, possibly linked to the fish receptor and/or fish cell context.

2. Materials and methods

2.1. Chemicals, materials and reagents

17β-Estradiol (E2, CAS#50-28-2), 17α-Ethinylestradiol (EE2, CAS#57-63-6), estrone (E1, CAS#53-16-7), estriol (E3, CAS#50-27-1), 2,4-dihydroxybenzophenone (BP1, CAS#131-56-6), 2,2′,4,4′-tetrahydroxybenzophenone (BP2, CAS#131-55-5), 2-hydroxy-4-methoxybenzophenone (BP3, CAS#131-57-7), 2,4,4′-trihydroxybenzophenone (THB, CAS#1470-79-7), diethylstilbestrol (DES, CAS#56-53-1), hexestrol (Hex, CAS#84-16-2), genistein (Gen, CAS#446-72-0), 4-*tert*-octylphenol (4OP, CAS#140-66-9), 4-nonylphenol (4NP, CAS#54181-64-5), bisphenol A (BPA, CAS#80-05-7), α-zearalenol (α-ZEE, CAS#36455-72-8), β-zearalenol (β-ZEE, CAS#71030-11-0) and α-zearalanol (α-ZEA, CAS#26538-44-3) were purchased from Sigma–Aldrich (France). All compounds were of purity higher than 98%. Stock solutions of chemicals were prepared in dimethyl sulfoxide (DMSO) at 10 mM and stored at −20 °C. Fresh dilutions of test chemicals were prepared before each experiment. Culture medium and additives were purchased from Gibco (France), fetal calf serum (FCS) and D-luciferin from Sigma–Aldrich (Quentin Fallavier, France). Cell culture plastics were obtained from BD Bioscience (France), except 96 well plates which were purchased from Greiner (France).

2.2. Plasmids

The construction of ERE-βGlob-Luc-SVNeo and pSG_5-rtER$_S$-puro plasmids that encode, respectively for the luciferase reporter gene and the rainbow trout estrogen receptor short form (rtERα), has been described previously by Balaguer et al. (1999) and Molina-Molina et al. (2008), respectively.

2.3. PLHC-1 cell line: culture conditions and stable transfection

The PLHC-1 cell line, obtained from the American Type Culture Collection (ATCC CRL 2406), is derived from the hepatocellular carcinoma of the topminnow *Poeciliopsis lucida* (Ryan and Hightower, 1994). PLHC-1 cells were routinely cultured at 30 °C in minimum essential medium with Earle's salts (E-MEM) supplemented with 10% v/v decomplemented fetal calf serum (FCS), 1% v/v non-essential amino acids, 1% v/v of sodium pyruvate, 50 U/ml of penicillin and streptomycin antibiotics in a 5% CO_2 humidified atmosphere. For stable transfection experiments, PLHC-1 cells were plated onto 100 mm diameter Petri dishes in complete E-MEM without antibiotics. Twenty four hours after plating, confluent cells were co-transfected with the two plasmids described above by using the Lipofectamine 2000™ reagent (Gibco, France), according to the manufacturer's instructions. After 3 h, transfection reagent was removed and cells were allowed to recover for 24 h before addition of 3 mg/ml G418 and 0.5 μg/ml puromycin as selecting agents. Medium was renewed every two days during one month before first clones were isolated and amplified. Only few resistant clones (about forty clones in three transfection dishes) were developed on the plates after one month of selection treatment with antibiotics. Nevertheless twenty clones could be isolated and tested for luciferase induction by E2. Among them, the clone 1.1 showed the highest induction of luciferase activity by E2. This clone was chosen for further experiments and called PELN-rtER for PLHC-1 *E*RE-*L*uciferase *N*eomycin-rtER.

2.4. Luciferase induction assay

PELN-rtER cells were seeded on 96 well plates (50,000 cells per well) in phenol red free medium supplemented with 3% dextran-coated charcoal treated FCS to remove serum steroids (DCC medium) and left to incubate for 24 h before chemical exposure. This medium was used to avoid interference due to estrogenic activity of phenol red and serum steroids in the assay. Solvent (DMSO) content did not exceed 0.1% v/v in the culture medium. Cells were exposed to test chemicals for 48 h at 25 °C. Luciferase activity was then determined in living cells as follows. The culture medium was removed and replaced by 50 μl of D-luciferin 0.3 mM in DCC medium. After 5 min allowing a stabilisation of the luminescent signal, luminescence counts were determined in a microplate luminometer (μBeta, Wallac). Results were expressed as percentage of maximal luciferase induced by E2, the reference ligand.

2.5. Vitellogenin assay in primary culture of rainbow trout hepatocytes (PRTH)

Adult male rainbow trout (*Onchorynchus mykiss*) were obtained from a local hatchery (INRA, Gournay-sur-Aronde, France). Fish were kept in tanks with aerated charcoal filtered tap-water at a temperature of 15 °C. Rainbow trout were fed with commercial fish food and acclimatized to laboratory conditions for a minimum of 2 weeks before use in the experiments. Hepatocytes were isolated as previously described (Laville et al., 2004) and seeded in 96 well Primaria™ microplates at a density of 5×10^5 cells per well and cultured at 15 °C in phenol red free Leibovitz-15 medium (L-15) supplemented with 5% DCC serum, penicillin and streptomycin (50 U/mL each) and 10 mM HEPES. Cells were left to incubate for 24 h before exposure to chemicals for 96 h. Solvent content (DMSO) did not exceed 0.1% v/v in the culture medium and half of the medium was renewed after two days with fresh medium containing the test chemical at the desired concentration. VTG quantification in extracellular culture medium was performed using a competitive enzyme-linked immunosorbent assay (ELISA) according to the method of Brion et al. (2002), using the AA-1 anti-salmon vitellogenin polyclonal antibodies (Biosense, Norway) and home-made standard VTG purified from E2-induced male rainbow trout (Brion et al., 2002).

2.6. Data analysis

A range of concentrations of chemical (0.01 nM–1 μM for estrogens and zearalenone metabolites and 1 nM–10 μM for the other chemicals) were tested in triplicate in each independent experiment. Data were expressed as mean value of relative luminescence units (RLU) ± standard deviation (SD). Dose–response curves were modeled by using the Regtox 7.5 Microsoft Excel™ macro (available at http://eric.vindimian.9online.fr/), which uses the Hill equation model and allows calculation of EC_{50}. Relative estrogenic potencies (REP) were determined as the ratio of 17β-estradiol EC_{50} to EC_{50} of the test chemical.

3. Results

3.1. Influence of temperature and exposure duration on luciferase induction by 17β-E2

The stable PELN reporter cell line was first examined for its ability to respond to the reference ER ligand E2 under different assay conditions. Since the functionality of rtERα has been shown to be sensitive to temperature (Matthews et al., 2002), we first tested effect of E2 at different temperatures. As seen in Fig. 1A, the EC_{50} va-