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Induction of vitellogenin synthesis in an Atlantic salmon (*Salmo salar*) hepatocyte culture: a sensitive *in vitro* bioassay for the oestrogenic and anti-oestrogenic activity of chemicals

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A variety of organic compounds have been documented to bind to the oestrogen receptor and induce oestrogenic effects in different vertebrates. The presence of these environmental oestrogens or oestrogen mimics in the aquatic environment has been suspected of disrupting the normal endocrinology of wild populations of fish. In this study, induction of vitellogenin synthesis in primary hepatocytes from Atlantic salmon (*Salmo salar*) was optimized and validated as an oestrogenic *in vitro* bioassay using a sensitive capture vitellogenin enzyme-linked immunosorbent assay. After proper optimization (cell media supplements, cell density, temperature and exposure time), this assay gave a sensitive and reproducible response to both endogenous steroids (relative potency: 17β-oestradiol ≫ oestriol > oestrone > 17α-oestradiol) and a range of common oestrogen mimics (relative potency: ethynyloestradiol and diethylstilboestrol ≫ genistein and zearalenone ≫ bisphenol A and 4-*t*-octylphenol > 4-*n*-nonylphenol and 2′-chloro,4-chloro-diphenyltrichloroethane (*o,p′*-DDT). However, the androgen testosterone and the putative oestrogen mimics dieldrin and toxaphene were not shown to be oestrogenic using this hepatocyte bioassay. Oestrogen-induced vitellogenin synthesis was efficiently inhibited by the anti-oestrogen ZM 189.154, suggesting that this bioassay may be used for testing both the oestrogenic and the anti-oestrogenic properties of chemicals.

Keywords: oestrogens, anti-oestrogens, xenoestrogens, hepatocytes, vitellogenin, Atlantic salmon, fish.

Introduction

A variety of organic compounds, introduced to the environment either accidentally or intentionally, have been documented to induce oestrogenic effects in fish (Sumpter and Jobling 1995). These environmental oestrogens or oestrogen mimics are able to bind to fish oestrogen receptors (Knudsen and Pottinger 1999, Tollefsen *et al.* 2002a) and stimulate oestrogen-mediated gene expression (Flouriot *et al.* 1995, Yadetie *et al.* 1999) as well as hepatic synthesis of the yolk protein precursor vitellogenin and eggshell (zona radiata) proteins *in vitro* (Pelissero *et al.* 1993, Celius *et al.* 1999) and *in vivo* (Sumpter and Jobling 1995, Arukwe *et al.* 1997b). Some of these chemicals have also been documented to interfere with oestrogen receptor (ER) regulation (Donohoe and Curtis 1996) as well as the biosynthesis (Freeman *et al.* 1984), metabolism (Arukwe *et al.* 1997a) and plasma

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binding (Tollefsen 2002, Tollefsen *et al.* 2002b) of endogenous steroids in fish. Contamination of natural waters by oestrogenic chemicals from industrial and municipal activities has consequently been proposed to cause widespread sexual disruption in wild populations of fish (Jobling *et al.* 1998).

Although there is mounting evidence to suggest that xenoestrogens may disturb endocrine function in fish, attempts to link adverse health effects in wild fish populations to exposure to oestrogenic compounds are complicated by the low potency of these chemicals. Recent data suggest that at least some of the oestrogenicity in natural waters may be ascribed to potent oestrogenic steroids of human origin (Desbrow *et al.* 1998). There is, however, little doubt that the xenoestrogens, which include compounds from distinctively different chemical groups and origins such as pharmaceuticals, pesticides and industrial compounds, as well as phytotoxins and mycotoxins, exist as complex mixtures in the environment. The urgent need to assess the ecological impact of environmental pollutants, either singly or in combination, has therefore prompted the development of sensitive bioassays for endocrine-disrupting effects.

The hepatic induction of vitellogenin (Vtg) synthesis in male and juvenile oviparous fish, which normally only occurs in maturing females following stimulation by 17β-oestradiol, has been proposed as a sensitive biomarker for oestrogenic chemicals of exogenous origin (Sumpter and Jobling 1995). The induction of this biomarker protein has been utilized as a suitable testing method for chemical screening and environmental monitoring *in vivo* (Arukwe *et al.* 1997b, Knudsen *et al.* 1997, Andersen *et al.* 1999).

Although *in vivo* testing of chemicals has become increasingly important in the risk assessment of oestrogenic chemicals, *in vitro* bioassays may still act as a useful tool for rapid and cost-efficient screening of chemicals. In the present paper, the induction of Vtg synthesis in an Atlantic salmon (*Salmo salar*) primary hepatocyte culture was optimized and validated as an *in vitro* bioassay for detecting oestrogenic (and anti-oestrogenic) chemicals.

Materials and methods

Chemicals

The test compounds 17β-oestradiol (E2), 17α-oestradiol (α-E2), oestrone (E1), oestriol (E3), testosterone (T), ethynyloestradiol (EE2), diethylstilboestrol (DES), genistein (GEN), zearalenone (ZEA), bisphenol A (BPA), dieldrin (DIN) and toxaphene (TOX) were all from Sigma (St Louis, Missouri, USA). The racemic 2′-chloro,4-chloro-diphenyltrichloroethane (*o,p′*-DDT) was a gift from Ciba-Geigy (Basel, Switzerland). 4-*t*-Octylphenol (OP) was obtained from Aldrich (Milwaukee, Wisconsin, USA) and 4-*n*-nonylphenol (NP) from Lancaster Synthesis Ltd (Morecambe, UK). The anti-oestrogen ZM 189.154 (ZM) was a kind gift from Dr T. Hutchinson (AstraZeneca, Brixham Environmental Laboratory, Brixham, UK). All the test chemicals had a minimum purity of 97%. Prior to use, all chemicals were diluted in methanol (99.8%) and stored at -80°C.

Fish

Saltwater-adapted male Atlantic salmon (~ 500 g) were obtained from the Norwegian Institute for Water Research (NIVA), Solbergstrand, Norway, and acclimatized to the experimental conditions (temperature $12 \pm 0.1^{\circ}$C, oxygen saturation $95 \pm 7\%$, salinity $32.5 \pm 0.5‰$, pH 8.1 ± 0.1, loading 10 g fish l^{-1}) for a minimum of 2 weeks prior to the experiments. The tanks received artificial illumination (100 lux) for 12 h a day. The salmon were fed daily with commercial salmon pellets (Felleskjøpet, Trondheim, Norway) in amounts corresponding to 1% of the total body mass.

Preparation of a monolayer culture

Hepatocytes were isolated using the two-step perfusion method of Berry and Friend (1969) as modified for fish by Andersson *et al.* (1983). At sampling, the fish was anaesthetized in benzocaine (75 mg l^{-1}, Fellesapoteket, Oslo, Norway) and killed by a blow to the head. The liver was then perfused *in situ* (10 ml min^{-1} for 10 min) with a calcium-free solution containing NaCl (7.14 g l^{-1}), KCl (0.36 g l^{-1}), $MgSO_4$ (0.15 g l^{-1}), Na_2HPO_4 (1.6 g l^{-1}), NaH_2PO_4 (0.4 g l^{-1}), $NaHCO_3$ (0.31 g l^{-1}) and ethylene glycol tetra-acetic acid (EGTA) (10 mg l^{-1}) at 12°C until all the blood had been washed out. The whitened liver was then perfused with the same buffer, now containing $CaCl_2$ (0.22 g l^{-1}) instead of EGTA, and 0.3 mg ml^{-1} collagenase (Sigma), for 5 min. After perfusion, the liver was removed and the cells dispersed in ice-cold calcium-free buffer (EGTA-buffer) containing 0.1% (w/v) bovine serum albumin (BSA) (Sigma). The cell suspension was filtered through a 100 μm nylon mesh and centrifuged at 50 *g* for 3 min at 4°C. The cells were then washed with ice-cold EGTA-buffer three times before being resuspended in serum-free L-15 medium containing l-glutamine; (0.29 g l^{-1}), $NaHCO_3$ (0.38 g l^{-1}), penicillin (100,000 Units l^{-1}), streptomycin (100 mg l^{-1}) and fungizone (0.25 g l^{-1}), all supplied by Biowhittaker Inc. (Walkersville, Maryland, USA). After viability and cell yield was measured using the trypan blue exclusion test, the cells were plated as a monolayer culture in 24-well Primaria® plates (Falcon, Becton Dickinson Labware, Oxnard, California, USA) and kept in an ambient atmosphere. Only cell preparations with a viability of 95% or more were used for the experiments. To properly optimize the method with regard to Vtg synthesis, the effect of serum supplements, incubation temperature, incubation time and cell density was assessed prior to the validation of the method. All glassware and instruments were autoclaved before use, and solutions were sterilized by filtration (0.22 μm).

Exposure of the hepatocytes

The cells were cultured for 1 day in unspiked medium prior to removal and replacement by medium containing the vehicle methanol (maximum 0.1% v/v) and different concentrations of test compounds, either alone or in combination with the anti-oestrogen ZM. The medium was changed after 2 days, and cells were re-exposed for additional 2-day periods before being removed and frozen at −80°C for subsequent analysis of Vtg. Vtg production was expressed either as absolute absorbance values (492 nm) or as Vtg production relative to the maximum synthesis obtained with E2 (100 nM). Cells were checked by visual inspection daily, and acute toxic effects on cells were determined using the trypan exclusion test at the end of the exposure period.

Analysis of Vtg

Vtg was measured by using a capture enzyme-linked immunosorbent assay (ELISA) essentially as described by Arukwe *et al*. (1997b). Undiluted cell medium was incubated in duplicates or triplicates in Maxisorp™ microtitre wells (Nunc, Roskilde, Denmark) overnight at 4°C. Non-specific binding was blocked with 200 μl of 2% (w/v) gelatine in phosphate buffered saline (PBS) (0.89 g l^{-1} Na_2HPO_4, 0.27 g l^{-1} KH_2PO_4, 8.77 g l^{-1} NaCl, pH 7.2) for 1 h at ambient temperature. For protein detection, 100 μl of a monoclonal mouse anti-salmon Vtg, BN-5 (1:2000, Biosense Laboratories, Bergen, Norway) diluted in 1% (w/v) gelatine-PBS was incubated in the wells at 37°C for 2 h. For enzymatic detection, a secondary monoclonal antibody towards mouse and rabbit immunoglobulin G (1:3000, Bio-Rad, Hercules, California, USA) was incubated at 37°C for 1 h, followed by the addition of the substrate *o*-phenylene-diamine dihydrochloride (Sigma Fast™, Sigma) at ambient temperature. The reaction was stopped by adding 50 μl 2 M H_2SO_4, and absorbance measured spectrophotometrically at 492 nm. Between all the incubation steps the plates were washed three times with PBS plus 0.05% (w/v) Tween-20, except prior to the addition of substrate, when the plates were washed five times.

Results

Optimization and validation of the bioassay

In a series of experiments, the cells were exposed to E2 under different assay conditions in order to optimize the sensitivity of the method. The results from these experiments show that the presence of fetal calf serum (FCS) (Biowhittaker Inc.) in the cell culture media influenced the measured level of Vtg (Figure 1). In comparison to serum-free media, the addition of 2% (v/v) of either heat-inactivated (65°C for 30 min), sex-steroid stripped (see Tollefsen 2002 for details) or untreated FCS reduced the measured level of Vtg considerably. The presence of FCS in the

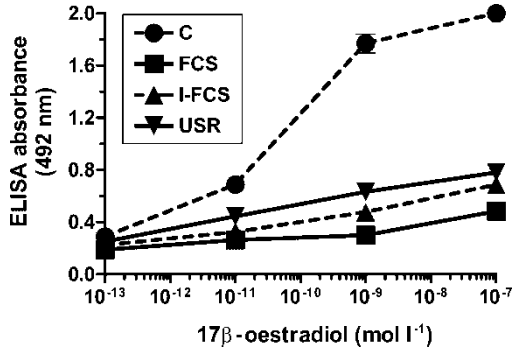

Figure 1. Capture ELISA analysis of Vtg in the growth media from an Atlantic salmon hepatocyte culture after 4 (2+2) days of exposure to 17β-oestradiol (E2) administered in serum-free media supplemented with 2% saline (C), 2% fetal calf serum (FCS), 2% heat-inactivated FCS (I-FCS) and 2% Ultrocer serum replacement (USR). Results given are the mean ELISA absorbance values (492 nm) ± SD for duplicate samples from one representative experiment.

cell media resulted in an apparent decrease in the maximum level produced, as well as a decrease in the sensitivity of the method. To test whether this decrease was due to inhibition of Vtg induction or interference with Vtg after synthesis, Vtg-containing cell culture media was co-incubated with different concentrations of FCS, Ultrocer serum replacement (Gibco BRL, Pasiley, UK) and BSA prior to ELISA analysis (Figure 2). The results from these experiments demonstrated that the presence of all supplements reduced the measured levels of Vtg in a dose-dependent and highly effective manner, thus indicating that media supplements interfere with the analysis of and not the synthesis of Vtg.

The cell density also seems to greatly influence the production of Vtg in the hepatocyte culture. In our experiments, a density-dependent increase in Vtg production was observed up to 500 000 cells ml^{-1} (1 ml/well), whereas higher densities than this led to drastic reductions in Vtg production (Figure 3A). A similar effect was observed when investigating the temperature dependency of Vtg

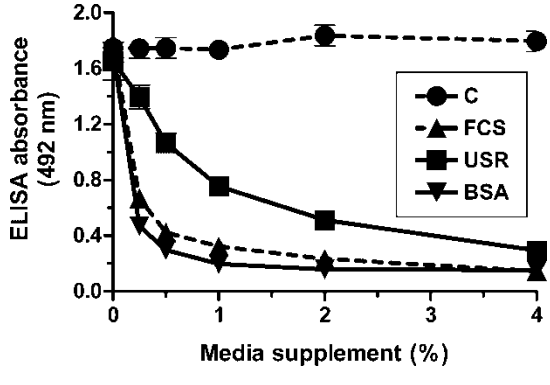

Figure 2. Capture ELISA analysis of Vtg in Vtg-enriched media after incubation (12 h) with growth media supplemented with saline (C), fetal calf serum (FCS), Ultrocer serum replacement (USR) and bovine serum albumin (BSA). Results given are the mean ELISA absorbance values (492 nm) ± SD for duplicate samples from one experiment.

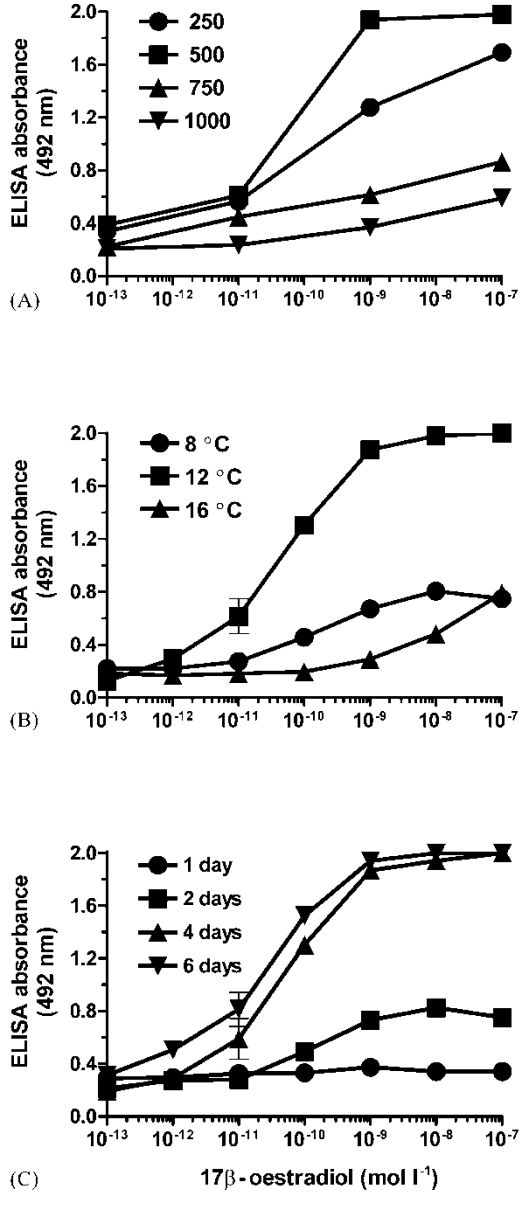

Figure 3. Capture ELISA analysis of Vtg in the growth media from an Atlantic salmon hepatocyte culture after exposure to 17β-oestradiol (E2) at: (A) different cell densities ($\times 10^3$ cells ml^{-1}, 1 ml, 2+2 days' exposure); (B) different incubation temperatures (500 000 cells ml^{-1}, 1 ml/well, 2+2 days' exposure); and (C) different exposure times (500 000 cells ml^{-1}, 1 ml/well, 12°C). Results given are the mean ELISA absorbance values (492 nm) ± SD for triplicate samples from one representative experiment.

production in the cell culture (Figure 3B). In these experiments, the optimal temperature was found to be around 12°C, whereas temperatures below and above this led to a profound decrease in Vtg production. To properly assess the effect of exposure time on Vtg synthesis, cells were also exposed to E2 for consecutive

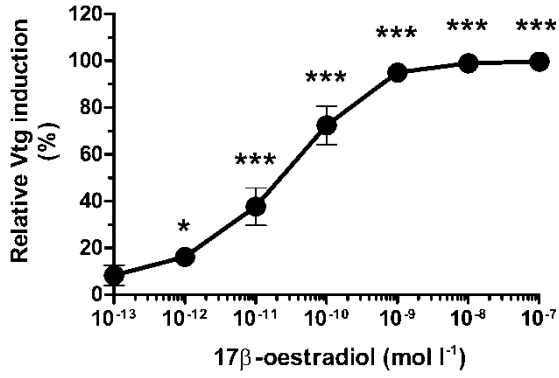

Figure 4. Capture ELISA analysis of Vtg in the growth media of an Atlantic salmon hepatocyte culture after 4 (2+2) days of exposure to 17β-oestradiol (E2) under optimal assay conditions. The results (mean ± SD, $n = 4$) are expressed relative to the maximum synthesis obtained for E2 (100 nM). $^{*}p < 0.05$; $^{***}p < 0.001$ compared with control group (one-way analysis of variance followed by Bonferroni's post test).

periods of 2 days (Figure 3C). Even though a significant increase in Vtg was detected after 48 h, 4 (2+2) days of exposure were required to obtain a high quality dose–response curve. Longer exposure to E2 was found to increase the sensitivity somewhat, but without improving the dose–response curve significantly. On the basis of these findings, the cells (500 000 cells ml^{-1}, 1 ml/well) were exposed for 4 (2+2) days in serum-free media at 12°C as the standard conditions for the bioassay.

The sensitivity and reproducibility of the bioassay was assessed by measuring the Vtg production after four individual exposures to a dilution series of E2 (Figure 4). The coefficient of variation across the concentration range was typically less than 15%, and a significant increase in Vtg production was routinely obtained at 1–10 pM E2. The maximum response (100%) was typically obtained at 10 nM E2.

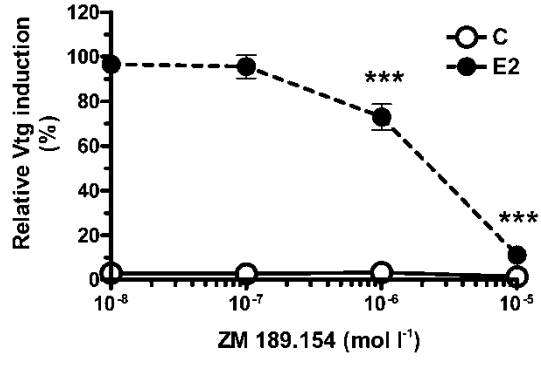

Figure 5. Capture ELISA analysis of Vtg in the growth media of an Atlantic salmon hepatocyte culture after 4 (2+2) days of exposure to different concentrations of the anti-oestrogen ZM 189.154 in combination with either the vehicle methanol (C) or 1 nM 17β-oestradiol (E2). The results (mean ± SD, $n = 2$) are expressed relative to the maximum synthesis obtained for E2 (100 nM). $p < 0.05$, $^{***}p < 0.001$ compared with exposure to 1 nM E2 alone (one-way analysis of variance followed by Bonferroni's post test).

Dilution of concentrations higher than this yielded an approximately linear relationship to the measured Vtg (results not shown), suggesting that the limiting factor to Vtg production was cellular constraints and not saturation of the microtitre wells. To verify that Vtg production was mediated through the ER, E2 (1 nM) were incubated in combinations with different concentrations of the anti-oestrogen ZM (Figure 5). The data from these experiments show that ZM was able to inhibit the production of Vtg in a dose-dependent manner.

The specificity of the bioassay was assessed by the ability of a range of steroids to induce the production of Vtg in the hepatocyte cell culture. The data presented in Figure 6 show that E2 was the most potent inducer of Vtg production in the hepatocyte culture, closely followed by E1 and E3. The concentrations of these minor oestrogens required to induce Vtg production to a similar extent as unlabelled oestradiol were typically 10-fold greater. The oestrogenic isomer α-E2 was considerably less potent than E2, whereas the androgen T failed to induce Vtg production. All of the oestrogens were able to induce Vtg production in a dose-dependent manner, although they were not able to reach maximum induction at the concentrations tested. Methanol, the vehicle used for the assays, was neither toxic nor able to induce production of Vtg in the concentration range used in the tests (data not shown). A summary of the relative potency of the steroids is given in Table 1.

Bioassay response to oestrogen mimics

Several putative oestrogen mimics, including naturally produced toxins (phytotoxins and mycotoxins) and anthropogenic chemicals (pharmaceuticals, pesticides and industrial compounds), were able to induce the production of Vtg in

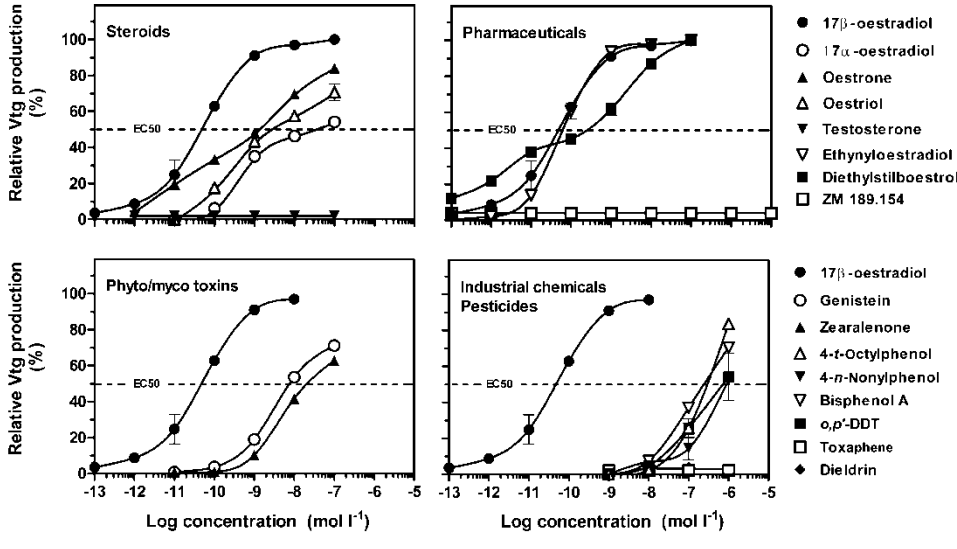

Figure 6. Capture ELISA analysis of Vtg in the growth media from an Atlantic salmon hepatocyte culture after 4 (2+2) days of exposure to a range of steroids, pharmaceuticals, phytotoxins/mycotoxins, industrial chemicals and pesticides. The results (mean±SD, $n = 2–4$) are expressed relative to the maximum synthesis obtained for E2 (100 nM).

Table 1. The mean (±SD) 50% effect concentration (EC_{50}) and relative oestrogenic potency (REP) for a range of steroids, pharmaceuticals, phytotoxins/mycotoxins, industrial chemicals and pesticides tested in an Atlantic salmon hepatocyte assay for oestrogenicity.

Chemical	n[a]	EC_{50} (mol l^{-1})	REP (%)[b]
Steroids			
17β-oestradiol (E2)	4	$2.6 \pm 1.6 \times 10^{-11}$	100
17α-oestradiol (α-E2)	2	$1.8 \pm 0.1 \times 10^{-8}$	0.14
Oestrone (E1)	2	$1.4 \pm 0.7 \times 10^{-9}$	1.8
Oestriol (E3)	2	$2.6 \pm 1.6 \times 10^{-9}$	1.0
Testosterone (T)	3	ND	–
Pharmaceuticals			
Ethynyloestradiol (EE2)	3	$6.2 \pm 0.1 \times 10^{-11}$	42
Diethylstilboestrol (DES)	4	$1.4 \pm 0.4 \times 10^{-10}$	19
ZM 189.154 (ZM)	3	ND	–
Phytotoxins/mycotoxins			
Genistein (GEN)	2	$1.1 \pm 0.2 \times 10^{-8}$	0.23
Zearalenone (ZEN)	2	$3.2 \pm 2.4 \times 10^{-8}$	8.1×10^{-2}
Industrial compounds			
4-*t*-Octylphenol (OP)	3	$2.9 \pm 0.3 \times 10^{-7}$	9.1×10^{-3}
4-*n*-Nonylphenol (NP)	3	$8.1 \pm 1.0 \times 10^{-7}$	3.2×10^{-3}
Bisphenol A (BPA)	3	$1.4 \pm 0.3 \times 10^{-7}$	1.7×10^{-2}
Pesticides			
2′-chloro,4-chloro-diphenyltrichloroethane (*o,p′*-DDT)	2	$7.2 \pm 1.1 \times 10^{-7}$	3.6×10^{-3}
Toxaphene (TOX)	3	ND	–
Dieldrin (DIN)	3	ND	–

[a] Number of individual determinations.
[b] Determined by comparing the concentration of test chemical required to induce 50% of the maximum cellular Vtg production (EC_{50}) to that for E2.
ND, no Vtg induction detected.

the hepatocyte culture (Figure 6). Most compounds tested showed a dose-dependent induction of Vtg, although only the most potent pharmaceuticals were able to reach the maximum induction obtained for E2 (100 nM). Of the environmental oestrogens tested, the oestrogenic pharmaceuticals EE2 and DES showed the highest potency, inducing Vtg production at about three to six times higher concentrations than required for E2. The phytoestrogen GEN and the mycoestrogen ZEA were approximately equally effective in inducing Vtg production. These naturally produced toxins were typically 500 times less potent than E2. However, most of the pesticides and industrial environmental oestrogens tested were considerably less potent in the bioassay than both oestrogenic pharmaceuticals and naturally produced toxins. In comparison, the industrial chemicals OP, NP and BPA, as well as the insecticide *o,p′*-DDT, were oestrogenic at concentrations about 10^4 times greater than E2. The pesticides DIN and TOX and the anti-oestrogen ZM were not able to induce any production of Vtg at the concentrations tested. The alkylphenols OP and NP, and *o,p′*-DDT were all acutely toxic to the hepatocytes at concentrations exceeding 10 μM when measured using the trypan blue exclusion test (data not shown). A summary of the relative potencies of the putative oestrogen mimics are given in Table 1.

Discussion

This study demonstrates that the detection of Vtg in the growth media from an Atlantic salmon primary hepatocyte culture using specific salmonid monoclonal antibodies can be used as an *in vitro* bioassay for the detection of oestrogenic chemicals. However, Vtg production was strongly dependent on the experimental conditions employed, thus making proper optimization important for the sensitivity and reproducibility of the assay. As seen with Vtg production *in vivo* (Korsgaard *et al.* 1986), *in vitro* production seems to be greatly limited at low temperatures. Although protein synthesis in general increases with temperature in fish (Jankowsky *et al.* 1981), an optimum was obtained at 12°C for Vtg synthesis in the hepatocyte monolayer culture when exposed to E2. In comparison, optimum Vtg production in a rainbow trout hepatocyte culture was obtained at a considerable higher temperature (20°C) using identical assay conditions (K.-E. Tollefsen, unpublished data). Furthermore, it was seen that Vtg was detected after only 2 days of exposure to E2, although proper dose–response curves were first obtained after 4 (2+ 2) days. The rapid induction of Vtg synthesis is in good agreement with the time course of the *in vivo* ER and Vtg mRNA upregulation seen in Atlantic salmon (Yadetie *et al.* 1999). With proper optimization, a highly sensitive and reproducible increase in the level of Vtg was routinely detected at picomolar concentrations of E2. This level of sensitivity is close to the levels found in the most common recombinant *in vitro* assays utilizing human ERs (Andersen *et al.* 1999). This is in contrast to the assumption that mammalian ERs exhibit considerably higher sensitivity towards oestrogens than fish (Le Dréan *et al.* 1995). The fact that Atlantic salmon has been reported to exhibit exceptionally high levels of hepatic ER among fish species (Mommsen and Lazier 1986), may explain the high level of sensitivity obtained. This is in accordance with the observation that Atlantic salmon hepatocytes induce Vtg synthesis at about a 10-fold lower concentration than rainbow trout (K.-E. Tollefsen, unpublished data), even though the ERs of the two species bind endogenous and exogenous oestrogens with similar affinity and specificity (Tollefsen *et al.* 2002a).

Interestingly, the sensitivity of the *in vitro* bioassay was reduced by 100- to 1000-fold when low concentrations of serum (FCS), which contain high concentrations of plasma proteins, were added. Although plasma proteins are known to bind specific steroids and some oestrogens with considerable affinity, and possibly modulate the bioavailable fractions of E2 (Tollefsen 2002, Tollefsen *et al.* 2002b), the major cause for the reduction in Vtg levels was apparently due to interference with the measurement of Vtg and not the cellular production of the protein. The fact that all media supplements tested were able to produce the same effect suggests that the presence of high concentrations of protein in the cell medium blocks the binding of Vtg to the ELISA wells or interferes with the antibody–antigen recognition in the ELISA analysis. This may in part explain the enhanced sensitivity of this bioassay compared with other *in vitro* fish bioassays utilizing Vtg ELISAs (Jobling and Sumpter 1993, Pelissero *et al.* 1993, Celius *et al.* 1999, Smeets *et al.* 1999, Okoumassoun *et al.* 2002). In fact, re-analysis of growth media from E2-induced cells that have been grown in the presence and absence of

2% FCS suggest that the problem is overcome by more robust sandwich ELISA assays (K.-E. Tollefsen, unpublished data).

The present study demonstrates that both endogenous sex steroids as well as naturally produced and synthetic compounds are able to induce the production of Vtg in a dose-dependent manner. Despite a 100- to 1000-times enhancement of bioassay sensitivity to both E2 and oestrogen mimics, the relative oestrogenic potencies compare well with similar bioassays from rainbow trout (Jobling and Sumpter 1993, Okoumassoun *et al.* 2002, Pelissero *et al.* 1993), Atlantic salmon (Celius *et al.* 1999) and common carp (Smeets *et al.* 1999). However, the main androgen T, which binds weakly to the Atlantic salmon ER (Tollefsen *et al.* 2002a), did not display any oestrogenic activity in our assay. Although T has been shown to activate the rainbow trout ER (Le Dréan *et al.* 1995) and to induce Vtg mRNA synthesis in rainbow trout hepatocytes at high concentrations (Mori *et al.* 1998), hepatocytes from Atlantic salmon were only responsive towards oestrogens at the concentrations tested. Furthermore, the bioassay was also able to detect the anti-oestrogenic activity of chemicals. As demonstrated with the anti-oestrogen ZM, a combined exposure of E2 and increasing concentrations of test chemicals may be used as a convenient test for anti-oestrogenicity. Although the number of known anti-oestrogenic chemicals is limited at present, the focus on alternative modes of action will prompt the development of bioassays capable of screening other endocrine-modulating properties of chemicals.

Interestingly, only a few of these compounds were able to induce the maximum synthesis obtained for oestradiol (100 nM). The reason for the lack of maximum induction is not immediately apparent, but reduced levels of Vtg in the cell media at high concentrations of test chemicals were well correlated to a reduction in cell viability measured using the trypan blue exclusion test (data not shown). This applied in particular to OP and NP, where results suggested that the limitation to oestrogenic activity observed at concentrations higher than 1 μM was due to toxic stress to the cells rather than the partial agonistic activity reported elsewhere (Soto *et al.* 1995). This is in accordance with the observation that weakly oestrogenic chemicals have a narrow window of effect in fish *in vitro* bioassays (Smeets *et al.* 1999).

Although a phylogenetic conservation of the ER has been proposed (Cox and Bunce 1999), comparative studies with piscine and mammalian ERs demonstrate that there exists a considerably interspecies difference in the amino acid sequence of the ligand-binding domain of the ER (Pakdel *et al.* 1989, Fielden *et al.* 1997). Consequently, the piscine ER may exhibit different ligand-binding preferences and/or affinities for oestrogen mimics than the human ER. This seems to be the case for the purported oestrogenic chemicals TOX and DIN, which were non-oestrogenic in our assay. These chemicals have previously been reported to bind to the rainbow trout ER and human ER, as well as to produce oestrogen-mediated growth of human breast cancer cells (Soto *et al.* 1995, Bolger *et al.* 1998, Matthews *et al.* 2000). The reason for this disagreement between results from different assays is not obvious, but interspecies differences in ER-activation sensitivity and specificity (Le Dréan *et al.* 1995, Matthews *et al.* 2000), multiple binding sites for ER activation (Arnold *et al.* 1996) and differences in biotransformation capability (Beresford *et*

al. 2000) have been proposed. Taking into account that DIN and TOX do not interact with the hepatic ER in Atlantic salmon and rainbow trout (Knudsen and Pottinger 1999, Tollefsen *et al.* 2002a), it seems highly unlikely that these chemicals act as oestrogens in salmonid fish via ER-mediated mechanisms at physiological relevant concentrations. In agreement with our results, a re-evaluation using *in vitro* and *in vivo* oestrogenic bioassays seems to confirm that TOX and DIN are not oestrogenic in mammalian species (Ramamoorthy *et al.* 1997).

Interestingly, GEN was found to be more potent than ZEN, which is in contradiction to their relative affinity to the Atlantic salmon ER (Tollefsen *et al.* 2002a) and their relative potency in some mammalian *in vitro* bioassays (Coldham *et al.* 1997, Zava *et al.* 1997). Recent work with the newly discovered human ER isoform (ER-β) have demonstrated that GEN acts as a full agonist for both ER-α and ER-β, whereas ZEA acts as a full agonist for human ER-α, and as a mixed agonist-antagonist for ER-β (Kuiper *et al.* 1998). Although several ER isoforms have been identified in fish (Hawkins *et al.* 2000), discrimination between the different isoforms was not performed in this study. The presence of multiple forms of the ER that are differentially expressed and activated in the hepatocytes may therefore possibly explain the difference between ER affinity and oestrogenic potency observed for Atlantic salmon. The relative potencies of other endogenous and exogenous oestrogens obtained in this study do, however, correlate well with their reported relative binding affinity to the Atlantic salmon ER (Figure 7), suggesting that binding to the ER governs the oestrogenic activity of the present set of test chemicals.

Conclusion

This study shows that Vtg synthesis in an Atlantic salmon primary hepatocyte culture can be used as a sensitive and reproducible bioassay for oestrogenic chemicals. The sensitivity of the bioassay was, however, highly dependent on the choice of experimental conditions (media supplements, cell density, and incubation temperature and time). However, both endogenous and exogenous oestrogens were

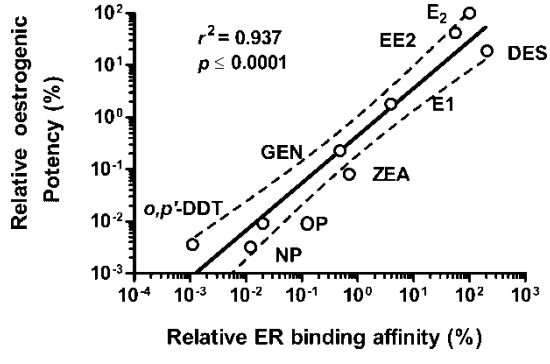

Figure 7. Comparison (regression line ± 95% confidence interval) between the relative binding affinity to a purified liver homogenate containing hepatic oestrogen receptors (ER) (data from Tollefsen et al. 2002a) and the relative oestrogenic potency in a hepatocyte culture from Atlantic salmon (data from Table 1).

able to induce dose-dependent Vtg synthesis after proper optimization of the assay. This induction occurred at substantial higher concentrations than required for E2, and the oestrogenic response for some of the environmental oestrogens seemed to be limited by acute toxic stress on the cells. The oestrogenic potency of the test chemicals was well correlated to the binding affinity to the Atlantic salmon ER, thus suggesting that oestrogenic potency was governed by binding to the ER.

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