A Sensitive Zonagenetic Assay for Rapid *in Vitro* Assessment of Estrogenic Potency of Xenobiotics and Mycotoxins

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Mounting evidence confirms that hepatic biosynthetic processes are essential for female sexual maturation in fish, which is directly controlled by estrogens. These oogenetic events (zonagenesis and vitellogenesis) are induced in both sexes by estrogens. In this paper, we report the induction of zona radiata (zr) proteins and vitellogenin in primary hepatocytes from Atlantic salmon (*Salmo salar* L.) exposed to xenoestrogens and mycotoxins. Cells were treated with doses of 1, 5, and 10 µM 4-nonylphenol (4-NP), *o,p′*-DDT, lindane (γ-HCH), and bisphenol A (BPA), which all induced zr proteins and vitellogenin in an approximate dose-dependent manner. Hepatocytes were also treated with combinations of xenoestrogens at 1 or 2 µM, resulting in elevated levels of both zr proteins and vitellogenin, compared to single treatment. The estrogenic activity of the mycotoxin zearalenone (ZEA) and its metabolites [α-zearalenol (α-ZEA) and β-zearalenol (β-ZEA)], with regard to zonagenesis and vitellogenesis, was assessed in this assay system. Mycotoxins were used at concentrations of 10, 100, or 1,000 nM. All induced zr proteins and vitellogenin, with α-ZEA being the strongest inducer. When cells were treated with xenoestrogens or mycotoxins in combination with an estrogen receptor inhibitor (ICI 182,780), the induction of both zr proteins and vitellogenin was inhibited in all cases. Thus, the reported estrogen effects are bonafide estrogen responses. Zona radiata proteins were more responsive than vitellogenin to both xenoestrogens and mycotoxins. The versatility and sensitivity of the hepatocyte assay demonstrates that biosynthesis of zr proteins provides a new supplementary method for estimating xenoestrogenicity and mycotoxin action. *Key words*: eggshell, environmental chemicals, hepatocytes, mycotoxin, zearalenone, zonagenesis, zona radiata proteins.
Environ Health Perspect 107:63–68 (1999). [Online 10 December 1998]
http://ehpnet1.niehs.nih.gov/docs/1999/107p63-68celius/abstract.html

During sexual maturation in fish, ovarian estrogen is transported in the blood to the liver, where it induces biosynthetic processes essential for the development of oocytes. These processes are induced in both sexes following estradiol treatment, which simplifies studies of xenoestrogenicity. Vitellogenin is a lipoglycophosphoprotein normally synthesized by the liver of female fish in response to 17β-estradiol (E_2) (*1,2*) and transported in the blood to the ovaries, where it serves as an energy source for the developing embryo (*3*). Like vitellogenin, synthesis of eggshell proteins (zona radiata proteins or zr proteins) is mediated by E_2 in the liver (*4*). Recently, we showed that hepatocytes from Atlantic salmon synthesize both vitellogenin and zr proteins when treated with E_2 *in vitro*, and importantly, zr proteins were more responsive than vitellogenin to E_2 (*5*). Furthermore, in fish exposed to environmental estrogens, zr proteins were induced differently than vitellogenin (*6,7*).

A number of environmental pollutants are known to disturb the endocrine system of animals, including fish and humans (*8*). These chemicals, often termed endocrine disruptors, may impair fertility in a wide range of wildlife species, including fish (*9*), amphibians (*10*), birds (*11*), and mammals (*12*). Several *in vitro* assays have been established to identify and assess suspected xenoestrogens. These include studies of estrogen receptor (ER) binding, cell proliferation, estrogen responsive element-regulated reporter genes, and yeast-based assays (*13*). Previous studies estimating the xenoestrogen activity of environmental chemicals measured the expression of vitellogenin in rainbow trout hepatocyte cultures (*14,15*). In such experiments it has been demonstrated that 4-nonylphenol (4-NP), octylphenol, *o,p′*-DDT, Aroclor 1221, and bisphenol A (BPA) induced vitellogenesis or vitellogenin expression (or production) and that a mixture of all chemicals elevated the level of vitellogenin compared to treatment with the chemicals singly (*16*).

In addition to synthetic chemicals, several toxins produced by fungi may have an adverse effect(s) on humans and animals. One of these is the mycotoxin zearalenone (ZEA), which is produced by different strains of *Fusarium*, a frequently encountered contaminant in cereals and other plant products (*17,18*). Katzenellenbogen et al. (*19*) reported that ZEA mycotoxins compete with estradiol for binding to the cytoplasmic ER. ZEA causes reproductive problems in farm animals due to its estrogenic activity (*17*), and in some animal models, ZEA is thought to be a primary initiator of hepatic tumors (*20*). ZEA undergoes metabolic reduction to the more estrogenic zearalenols (*21*). The estrogenic action greatly depends on the stereochemical (α, β) configuration of the hydroxyl group in the nonaromatic moiety of their structures (Fig. 1), and α-ZEA is about three times more estrogenic than ZEA (*22*). It is of interest to test ZEA in a fish model since a previous study reported a reduced number and quality of spermatozoa in carp following ZEA exposure (*23*).

Hepatocytic *in vitro* synthesis of vitellogenin provides a useful and rapid assessment of xenoestrogenic potentials (*16*). However, zr proteins were recently found to be more sensitive to E_2 (*5*) and xenoestrogens than vitellogenin (*6,7*). The availability of specific antibodies to oocytic proteins has enabled documentation, both *in vivo* and *in vitro*, of initiation of oogenesis after either estrogenic or xenoestrogenic induction. To our knowledge, the relative potency of ZEA and its α and β metabolites has so far not been directly evaluated. Thus, it is of interest to compare their estrogenic potentials with respect to zr protein biosynthsis. *In vitro* estrogenic potencies of 4-NP, *o,p′*-DDT, BPA, lindane (γ-HCH), and ZEA mycotoxins (see Fig. 1) were studied using primary hepatocyte cultures from Atlantic salmon. The aim of this study was to evaluate the relative merits of zr proteins and vitellogenin biosynthesis in assessing the estrogenic potentials of environmental chemicals and mycotoxins by an *in vitro* cellular assay.

Materials and Methods

Chemicals. 4-Chloro-1-naphthol tablets, *o*-phenylenediamine dihydrochloride (OPD), EGTA, HEPES, bovine serum albumin (BSA), ZEA, α-ZEA, β-ZEA, and BPA were purchased from Sigma Chemical Company (St. Louis, MO). *o,p′*-DDT was purchased from Promochem AB (Ulricehamn, Sweden); 4-NP (85% *p*-isomers) was from Fluka, (Buchs, Switzerland), and lindane was from Riedel-deHaën (Seelze, Germany).

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We thank B. Vose at ZENECA Pharmaceuticals for the support of ICI 182,780. We thank A. Goksøyr and A. Arukwe for help and comments. This study was supported by the Norwegian Research Council, Programme for Ecotoxicology.
Received 20 May 1998; accepted 18 August 1998.

Other chemicals and secondary antibodies for ELISA analysis were from Bio-Rad Laboratories (Hercules, CA). The monoclonal antibody against salmon vitellogenin (BN-5 and KB-1) was purchased from Biosense Laboratories (Bergen, Norway). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, and antibiotic/antimycotic solution (AAS) were obtained from Gibco BRL (Life Technologies Ltd., Paisley, UK). Primaria plates for cell cultures were from Falcon (Becton Dickinson Labware, Oxnard, CA). ICI 182,780 was a gift from B. Vose, Zeneca Pharmaceuticals (Macclesfield, UK). Other chemicals were of highest commercial purity.

Collagenase perfusion of liver. Hepatocytes were isolated from male or juvenile Atlantic salmon (Sævareid fish farm, Fusa, Norway) by a two-step perfusion method of Berry and Friend (*24*), as modified for fish by Andersson et al. (*25*). The liver was perfused using a peristaltic pump at a flow rate of 22 ml/min. First, the liver was perfused *in situ* for 10 min with a calcium-free solution containing NaCl (7.14 g/l), KCl (0.36 g/l), $MgSO_4$ (0.15g/l), Na_2HPO_4 (1.6 g/l), NaH_2PO_4 (0.4 g/l), $NaHCO_3$ (0.31 g/l), and EGTA (20 mg/l) at ambient temperature until all blood had been washed out. The whitened liver was then perfused *in situ* for approximately 10 min in the same buffer, but containing calcium (0.22 g/l) instead of EGTA, and collagenase 80 mg/150 ml. All glassware and instruments were autoclaved before use. Solutions were sterilized by filtration using 0.22 µm Millipore filter (Millipore AB, Sundbyberg, Sweden).

Preparation and treatment of hepatocyte cultures. After perfusion, the liver was dispersed in the calcium-containing buffer supplemented with BSA (0.1 g/100 ml). The suspension was filtered through a 150-mm nylon monofilament filter and centrifuged at 50*g* for 2 min. Cells were washed with medium three times and finally resuspended in medium. Viability of hepatocytes used for experiments was always over 90%, as determined by Trypan blue exclusion. After measuring cell yield, cells were plated on 24-well multiwell Primaria plates (≈0.5×10^6 cells per well) in DMEM (without phenol red) containing 0.5% (v/v) FBS, 15 mmol/l HEPES, glutamine (0.3 g/l medium) and AAS (1,000 units/l pencillin, 1 µg/ml streptomycin, and 0.75 µg/ml amphotericin). Cells were kept at a constant temperature of 10.0°C (± 0.5) in ambient atmosphere. After plating, the cells were cultured for 2 days in medium without test compounds. Medium was then removed and replaced by medium containing environmental chemicals or mycotoxins. Medium was changed every day for 4 days. Cells were treated with different doses of the test chemicals 4-NP, *o,p'*-DDT, γ-HCH, and BPA, all in concentrations of 1, 5, and 10 µM. Hepatocytes were treated with each chemical alone (5 µM) or in combination with the ER inhibitor ICI 182,780 (1 µm). Cells were also treated with a mixture of all chemicals in concentrations of 1 µM (mixture 1) and 2 µM (mixture 2) alone and in combination with 1 µM ICI 182,780. Furthermore, hepatocytes were treated with the mycotoxin

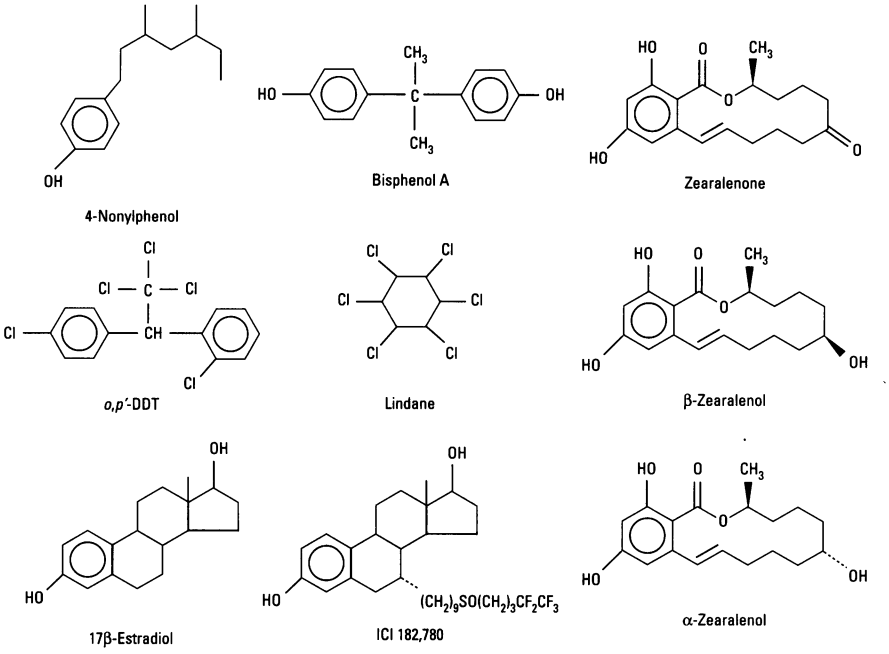

Figure 1. Chemical structures of the different compounds used in the study.

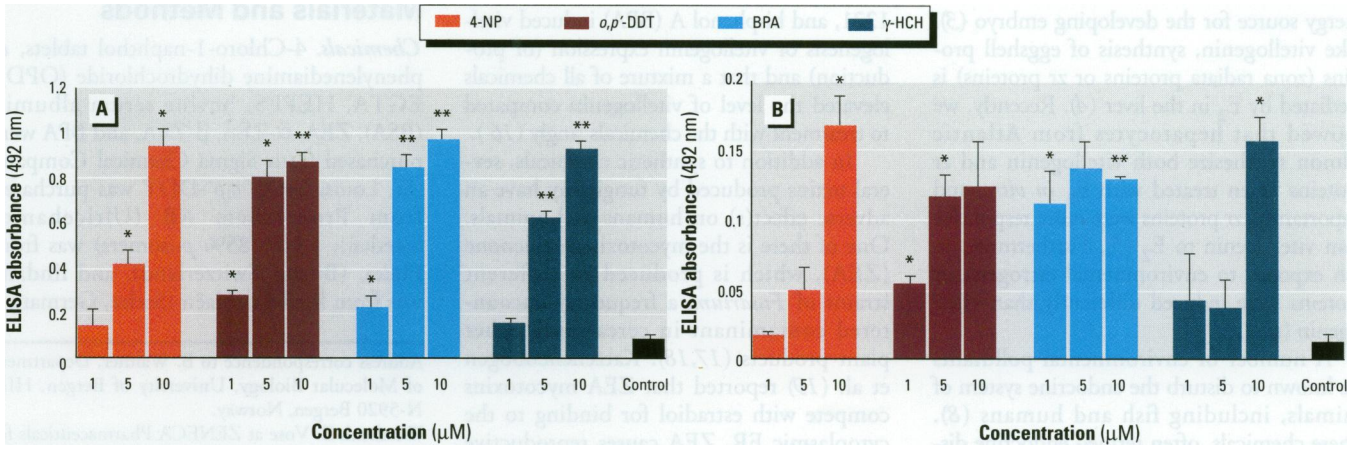

Figure 2. Induction of (A) zona radiata proteins and (B) vitellogen in hepatocytes after treatment with 1, 5, and 10 µM 4-nonylphenol (4-NP), *o,p'*-DDT, lindane (γ-HCH), or bisphenol A (BPA) as measured by the enzyme-linked immunosorbent assay (ELISA). Zona radiata proteins were measured after 48 hr exposure, and vitellogenin was measured after 96 hr exposure. Data are given as means ± standard deviations of triplicate cell treatments.
*$p<0.01$; **$p<0.001$.

ZEA and its metabolites, α-ZEA and β-ZEA, alone or in combination with ICI 182,780. Hepatocytes were treated with increasing concentrations: 10, 100, and 1,000 nM of each compound. When cells were incubated with ICI 182,780 in combination with mycotoxins, a concentration of 1 μM of the inhibitor was used with 100 nM α-ZEA or with 1,000 nM ZEA or β-ZEA. All compounds were solubilized in absolute ethanol, and the amount of ethanol in medium did not exceed 0.1% of total volume. Control cells were treated with medium containing 0.1% ethanol. Cell treatment studies were repeated in triplicate, and experiments were repeated with primary cell preparations from three fish.

Antiserum preparation and specificity. Antiserum to zr proteins from Atlantic salmon was prepared according to Rong (*26*) as follows: zr proteins collected from plasma were purified by preparative SDS polyacrylamide gel electrophoresis (PAGE), and a polyclonal antiserum that recognized all three zr proteins was raised in rabbit. The specificity of the polyclonal antiserum was checked by Western blotting, and no cross-reactivity with purified vitellogenin or other plasma proteins was seen (*4,27*).

ELISA analysis. The enzyme-linked immunosorbent assay (ELISA) was based on the method of Oppen-Berntsen et al. (*28*), except that nonspecific protein binding to the wells was blocked using 2% BSA (200 μl). The wells were incubated with 100 μl polyclonal antiserum against zr proteins, diluted 1:1000 in 1% BSA, or 100 μl monoclonal antibody against vitellogenin (KB-1 or BN-5) at a concentration of 1:500 in 1% BSA. Secondary antibodies were goat anti-rabbit IgG horseradish peroxidase conjugate (1:3000) and goat anti-mouse IgG horseradish peroxidase (1:3000). Absorbance was measured at 492 nm. Samples containing all reagents except antigen were used to measure background values of the antibodies. This absorbance value was then subtracted from all measurements. *p*-Values were calculated by comparing each treatment to control (*t*-test; Microsoft Excel 5.0; Microsoft, Redmond, WA).

Results

Hepatocytes were treated with increasing amounts (1, 5, and 10 μM) of 4-NP, *o,p'*-DDT, γ-HCH, or BPA. All chemicals induced zr proteins in an approximate dose-dependent manner after 48 hr of treatment (Fig. 2A). At this time of exposure vitellogenin was not detected. However, after 96 hr of treatment, vitellogenin did show a delayed but similar dose-dependent induction (Fig. 2B).

Figure 3 presents the induction of hepatocytic production of zr proteins and vitellogenin after *in vitro* treatment with different xenobiotics. A time-dependent induction of both zr proteins and vitellogenin was found when cells were treated with 5 μM of either 4-NP, *o,p'*-DDT, γ-HCH, or BPA. Zr proteins were detected in the culture medium 48 hr before vitellogenin.

Figure 4 shows the effect of mixtures of the various compounds compared to individual treatment. Analysis of zr proteins in medium after 48 hr of exposure showed elevated levels of zr proteins when cells were treated with mixtures of chemicals compared to treatment with individual compounds (Fig. 4A). Additionally, the level of zr proteins was elevated after treatment with the 2 μM mixture of chemicals, as compared to treatment with the 1 μM mixture of the same chemicals. However, after 96 hr exposure, the response was maximal both for *o,p'*-DDT and BPA alone, so that treatment of cells with mixtures of 1 μM or 2 μM appears not to elevate the levels of zr proteins in terms of a relative ELISA response (Fig. 4B). Vitellogenin was not induced after 48 hr of exposure. However, when medium was analyzed after 96 hr, induction of vitellogenin was elevated after treatment with both 1 μM and 2 μM mixtures, compared to individual treatment (Fig. 4C). Additionally, treatment of hepatocytes with a mixture of 2 μM of all chemicals resulted in stronger induction than was seen for cells treated with the 1 μM mixture (Fig 4C).

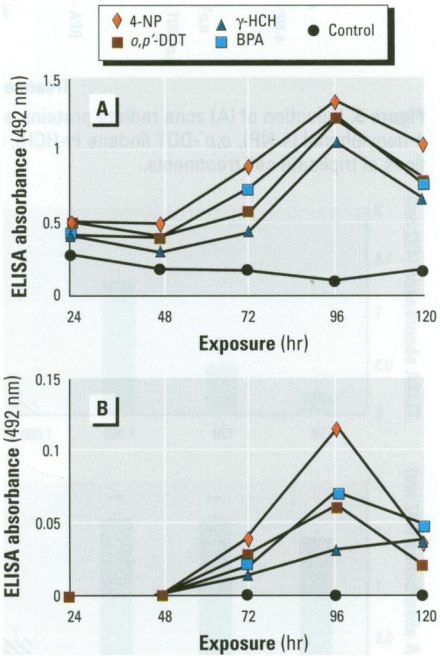

Figure 3. Time-course of induction of (A) zona radiata proteins and (B) vitellogenin after treatment of hepatocytes with 5 μM 4-nonylphenol (4-NP), *o,p'*-DDT, lindane (γ-HCH), or bisphenol A (BPA). Media were sampled and analyzed by enzyme-linked immunosorbent assay (ELISA) every 24 hr. Data are given as means of triplicate cell treatments.

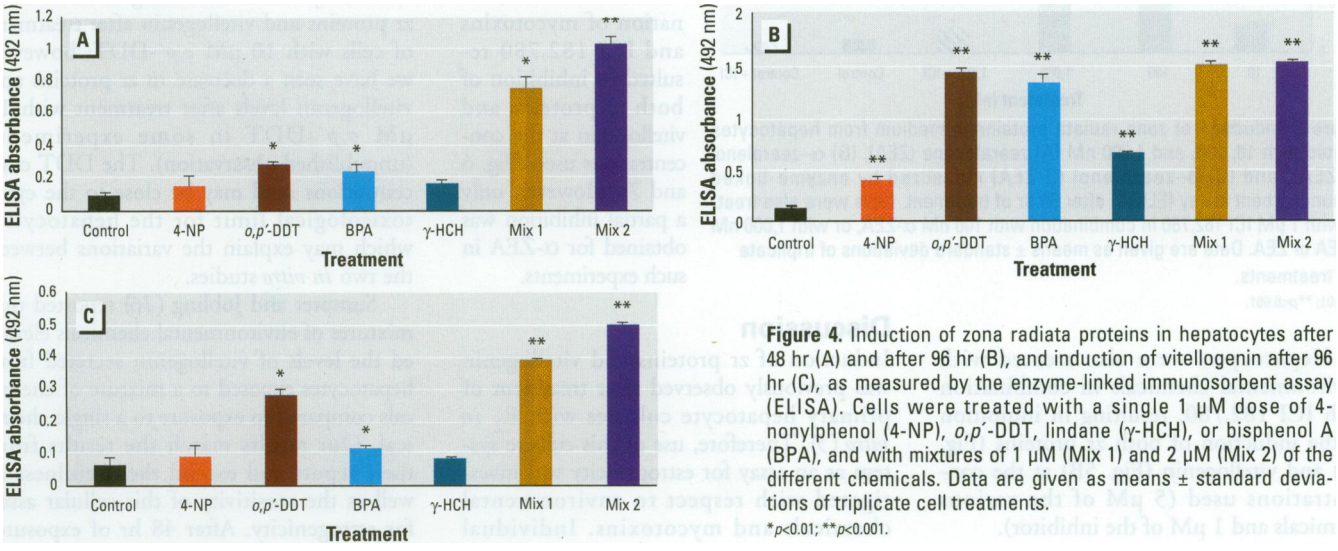

Figure 4. Induction of zona radiata proteins in hepatocytes after 48 hr (A) and after 96 hr (B), and induction of vitellogenin after 96 hr (C), as measured by the enzyme-linked immunosorbent assay (ELISA). Cells were treated with a single 1 μM dose of 4-nonylphenol (4-NP), *o,p'*-DDT, lindane (γ-HCH), or bisphenol A (BPA), and with mixtures of 1 μM (Mix 1) and 2 μM (Mix 2) of the different chemicals. Data are given as means ± standard deviations of triplicate cell treatments.
*$p<0.01$; **$p<0.001$.

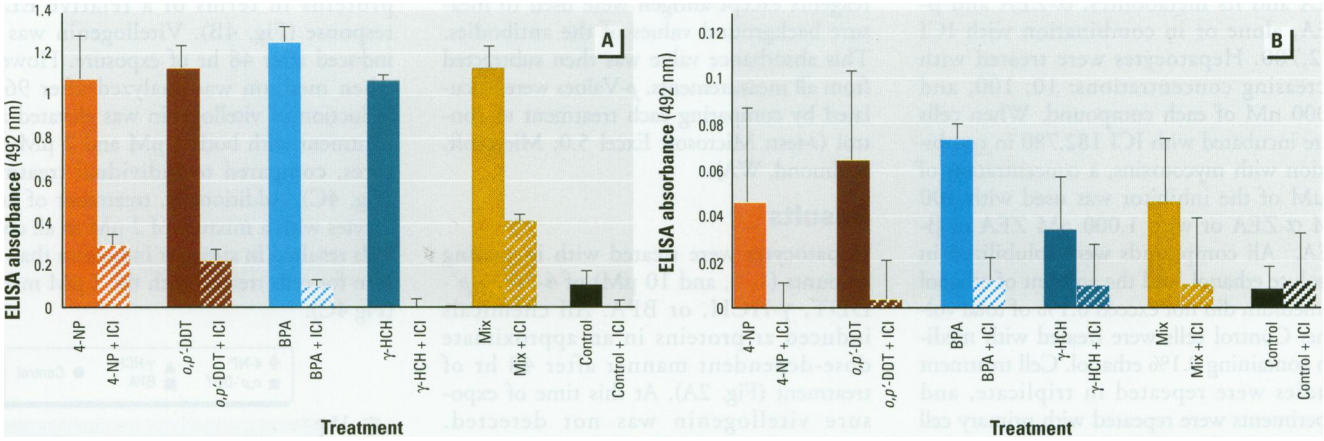

Figure 5. Induction of (A) zona radiata proteins and (B) vitellogenin measured by enzyme-linked immunosorbent assay (ELISA) after 96 hr of treatment with 5 µM 4-nonylphenol (4-NP), *o,p'*-DDT lindane (γ-HCH), or bisphenol A (BPA) alone or in combination with 1 µM ICI 182,780. Data are given as means ± standard deviations of triplicate cell treatments.

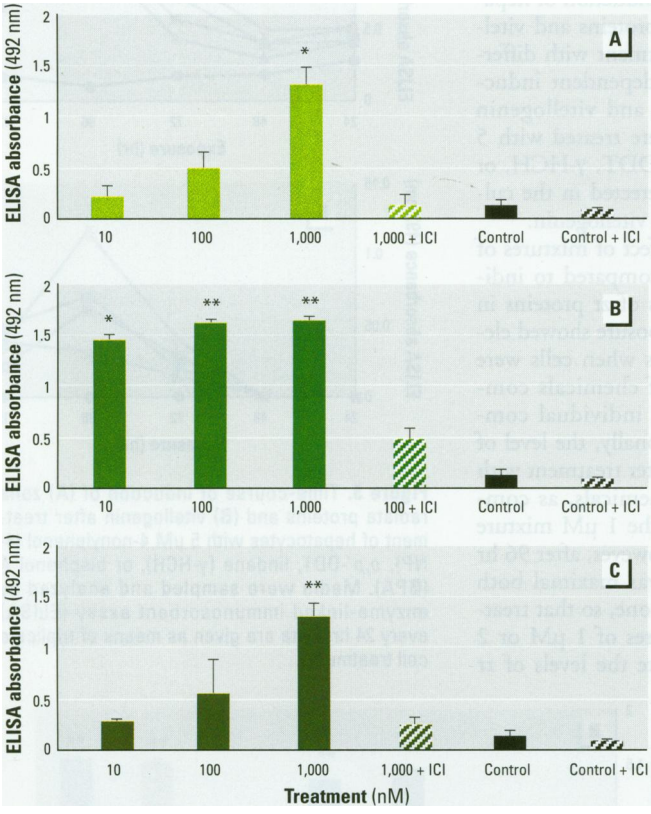

Figure 6. Induction of zona radiata proteins in medium from hepatocytes treated with 10, 100, and 1,000 nM (A) zearalenone (ZEA), (B) α–zearalenol (α-ZEA), and (C) β–zearalenol (β-ZEA) measured by enzyme-linked immunosorbent assay (ELISA) after 96 hr of treatment. Cells were also treated with 1 µM ICI 182,780 in combination with 100 nM α-ZEA, or with 1,000 nM β-ZEA or ZEA. Data are given as means ± standard deviations of triplicate cell treatments.

*$p<0.01$; **$p<0.001$.

Hepatocytes were also treated with environmental chemicals in combination with ICI 182,780, resulting in inhibition of the induction of both zr proteins (Fig. 5A) and vitellogenin (Fig. 5B) at the concentrations used (5 µM of the various chemicals and 1 µM of the inhibitor).

Cells were treated with ZEA and its two metabolites, α-ZEA and β-ZEA, in 10, 100 and 1,000 nM concentrations. α-ZEA was a strong inducer of both zr proteins (Fig. 6) and vitellogenin (Fig. 7) measured at 96 hr exposure. Vitellogenin was induced in a dose-dependent manner in contrast to zr proteins, which showed similar (almost maximal) induction at the lowest concentration of α-ZEA. ZEA and β-ZEA also induced synthesis of zr proteins and vitellogenin, but to a lower extent. Hepatocytes treated with a combination of mycotoxins and ICI 182,780 resulted in inhibition of both zr proteins and vitellogenin at the concentrations used (Fig. 6 and 7). However, only a partial inhibition was obtained for α-ZEA in such experiments.

Discussion

Induction of zr proteins and vitellogenin was previously observed after treatment of primary hepatocyte cultures with E_2 *in vitro* (*5*). Therefore, use of this culture system as an assay for estrogenicity was investigated with respect to environmental chemicals and mycotoxins. Individual xenobiotics exhibited varying estrogenicity as assessed by this assay, and the results show that zr proteins were more responsive than vitellogenin to both xenoestrogens and mycotoxins. Importantly, ICI 182,780 inhibited synthesis of both zr proteins and vitellogenin in hepatocytes induced by any of the tested environmental chemicals (singly or in a mixture) or mycotoxins. Hence, this assay system measures estrogenicity of chemicals.

Results from the present work correspond with previous studies showing dose-dependent induction of vitellogenin, when hepatocytes from rainbow trout were treated with increasing amounts of 4-NP, octylphenol, Aroclor 1221, or BPA (*16*). Sumpter and Jobling (*16*) reported that hepatocytes treated with 1 and 5 µM *o,p'*-DDT had increasing levels of vitellogenin, while a dose of 10 µM decreased the level significantly (*16*). They concluded that 10 µM *o,p'*-DDT was cytotoxic to the cells. Our study shows increasing levels of both zr proteins and vitellogenin after treatment of cells with 10 µM *o,p'*-DDT; however, we have seen a decrease in zr proteins and vitellogenin levels after treatment with 10 µM *o,p'*-DDT in some experiments (unpublished observation). The DDT concentrations used may be close to the cytotoxicological limit for the hepatocytes, which may explain the variations between the two *in vitro* studies.

Sumpter and Jobling (*16*) reported that mixtures of environmental chemicals elevated the levels of vitellogenin secreted from hepatocytes exposed to a mixture of chemicals compared to exposure to a single chemical. Our results match the results from their report and extend the usefulness as well as the sensitivity of this cellular assay for estrogenicity. After 48 hr of exposure,

hepatocytes treated with mixtures of chemicals (1 µM or 2 µM) had elevated levels of zr proteins compared to those treated with a single compound. In contrast, no vitellogenin was detected at this time (see Fig. 3). However, the level of vitellogenin after 96 hr of exposure did exhibit approximate dose dependency with respect to the compounds tested and also showed higher induction when cells were treated with a mixture of all the chemicals at a concentration of 2 µM, compared to a mixture of 1 µM of each compound. The zr protein antiserum in the ELISA analysis gave a higher response than the vitellogenin antibody. However, our previous studies revealed a concentration-dependent and distinct temporal induction of zr proteins and vitellogenin when salmon was exposed to increasing E_2 amounts, both *in vivo* and *in vitro* (*5*). Furthermore, our recent studies of levels of zr protein mRNA and vitellogenin mRNA in liver samples from salmon injected with increasing amounts of E_2 showed that zr protein mRNA was induced at lower levels of E_2 than vitellogenin mRNA (*29*); this corresponds with the absence or presence of the respective proteins in the assay medium observed in the present study.

The *in vitro* findings for zr proteins and vitellogenin at 48 hr and 96 hr exposure, respectively, are somewhat difficult to compare with previous results from *in vivo* experiments (*30*). Arukwe et al. (*30*) reported that *o,p'*-DDT, BPA, γ-HCH, and Aroclor 1254 injected into Atlantic salmon in combination with 4-NP did not elevate the levels of zr proteins or vitellogenin after 2 weeks of exposure when compared to a single treatment with 4-NP or a mixture of all the chemicals. However, these *in vivo* observations are interpretable in terms of the present results for zr proteins at 96 hr of exposure. Discrepancies between *in vivo* and *in vitro* experiments could be due to exposure time, xenobiotic metabolism, or other factors. However, both studies demonstrate the complexity of pinpointing synergistic or additive effects of environmental chemicals.

ICI 182,780 is a potent and specific inhibitor of estrogen action, with a relative binding affinity of 0.89, compared to 1.0 for E_2 (*31*). In our study, ICI 182,780 inhibited synthesis of both zr proteins and vitellogenin when combined with environmental chemicals (individually or in a mixture) or mycotoxins. Induction of zr proteins was only partially inhibited when cells were treated with α-ZEA in combination with ICI 182,780. A similar result was seen when hepatocytes were treated with high concentrations of E_2 in combination with inhibitor (data not shown). ICI 182,780 is a competitive inhibitor, and incomplete inhibition may be due to the strong estrogenicity of the α-ZEA at the concentration used in the present study. Mechanisms of estrogenic action of xenoestrogens are complex, but these results indicate that the ER is involved in this cellular assay system. Previous studies (*32*) using the ER inhibitors tamoxifen and ICI 164,384 showed that synthesis of both vitellogenin mRNA and ER were inhibited when rainbow trout hepatocytes were treated with E_2, lindane, Aroclor 1254, 4-NP, or chlordecone in combination with ER inhibitors.

The present paper demonstrates for the first time the relative estrogenic effect of ZEA and its metabolites with regard to both zonagenesis and vitellogenesis. The estrogenic potential of ZEA has been assessed *in vivo* by uterotropic assays in rodents (*33*) and *in vitro* in human cancer cell lines such as MCF-7 cells (*34*); receptor binding studies in, for example, a HeLa cell co-transfection assay (*35*); and genetically transformed mouse cells (*36*). The general conclusion from these studies is that ZEA competes with E_2 in the binding to the cytosolic ER. Our results are in accordance with previous receptor binding studies in terms of the relative affinity of ZEA versus its biologically active metabolites. The observation that α-ZEA is more potent than ZEA, which in turn is somewhat more potent than β-ZEA, agrees well with their respective affinities to ER (*37,38*). To our knowledge, this is the first time the estrogenic response of ZEA and its metabolites have been directly assessed *in vitro*. Our study shows that this assay is capable of detecting estrogen activity of compounds with widely differing chemical structure. As seen in Figure 1, chemical structures of the compounds used in our study are dissimilar; however, some functional groups are more important then others for estrogenic activity. For example, the *para*-substituted phenolic group is important according to Jordan et al. (*39*).

While human-oriented systems are helpful in assessing potential ecological impact of environmental chemicals, few of the available *in vitro* assays have specifically focused on wildlife as opposed to human risk aspects. Versatile *in vitro* assays are valuable for reproductive toxicology. *In vitro* assays are relatively inexpensive compared to most *in vivo* experiments and are often faster to perform. Zr protein and vitellogenin *in vitro* assays are useful to assess exposure, as well as to study possible mechanisms of action of xenoestrogens. This study emphasizes the usefulness of zr proteins in this respect, and there are some indications that zr proteins may be more generally present in other vertebrates (*40*).

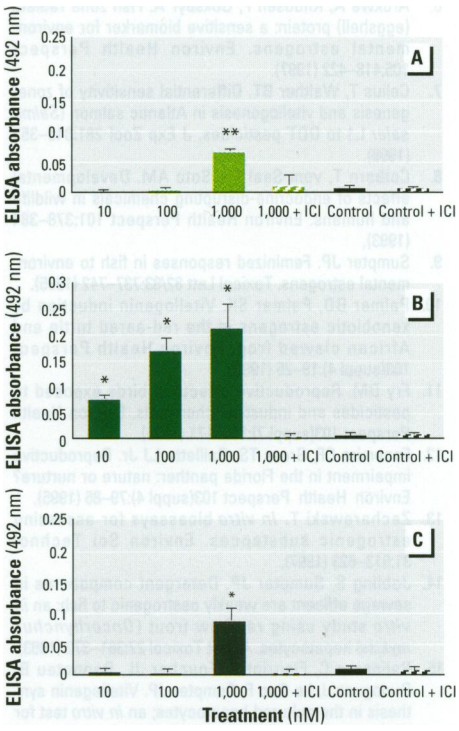

Figure 7. Induction of vitellogenin in medium from hepatocytes treated with 10, 100, and 1,000 nM (A) zearalenone (ZEA), (B) α-zearalenol (α-ZEA), and (C) β-zearalenol (β-ZEA), measured by enzyme-linked immunosorbent assay (ELISA) after 96 hr of treatment. Cells were also treated with 1 µM ICI 182,780 in combination with 100 nM α-ZEA, or with 1,000 nM β-ZEA or ZEA. Data are given as means ± standard deviations of triplicate cell treatments.
*$p<0.01$, **$p<0.001$.

In vivo assays provide information about net effects in whole animals, where xenobiotics may act at multiple sites and organs, while revealing little about the mechanisms involved. A combination of *in vivo* experiments and *in vitro* assays is optimal to delineate both mechanisms and effects of xenobiotics as a part of understanding realistic risks and gross impact of environmental chemicals.

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