Effects of Primary Exposure to Environmental and Natural Estrogens on Vitellogenin Production in Carp (Cyprinus carpio) Hepatocytes

T. Rouhani Rankouhi,*1 I. van Holstein,† R. Letcher,‡ J. P. Giesy,‡ and M. van den Berg∗

*Institute for Risk Assessment Sciences (IRAS), Utrecht University, P. O. Box 80176, 3508 TD, Utrecht, The Netherlands; †Great Lakes Institute for Environmental Research (GLIER), University of Windsor, Windsor, Ontario, Canada; and ‡Department of Zoology, National Food Safety and Toxicology Center, Institute of Environmental Toxicology, Michigan State University, East Lansing, Michigan 48824

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Vitellogenin (vtg) is a precursor of the yolk proteins lipovitelline and phosvitin and is synthesized as a consequence of estrogen-dependent gene expression in female and male hepatocytes of egg-laying vertebrates. Freshly isolated carp hepatocytes of a genetically uniform strain of adult male carp (Cyprinus carpio) were used to investigate the effects of primary exposure to estrogenic compounds on the vitellogenic response to xenoestrogens. Isolated carp hepatocytes were first exposed (primary exposure) to 50 or 100 μM of either methoxychlor (MXCL) or bisphenol A (BPA), different concentrations of estrone (E1; 1 or 10 nM) or 17β-estradiol (E2; 0.1 or 1 nM) for 2 days. Hepatocytes were exposed to xenoestrogens (secondary exposure) at both 2 and 5 days after isolation. Hepatocytes were cultured for a total period of 8 days. A competitive indirect ELISA was used to determine the level of vtg after 8 days. The concentration of chemicals used for the primary exposure induced vtg to a level that was less than 10% of the response elicited by E2 (1000 nM). A cytotoxic response, measured by MTT, was not observed after primary exposure to any of the xenoestrogens. After primary exposure to MXCL, vtg production in response to E2 was increased by 4-fold, and vitellogenesis in response to E1 treatment was doubled compared with vitellogenesis without pretreatment. No significant differences were observed between primary exposure to 50 and 100 μM MXCL. Primary exposure to 50 and 100 μM BPA increased the maximum vtg production in response to secondary E2 exposure by about 5- and 7-fold, respectively. Primary exposure to BPA (50 and 100μM) followed by secondary exposure to E1 showed a 4- and 5-fold increase of the vtg synthesis in comparison to E1 exposure alone. Primary exposure to the endogenous estrogens had no significant influence on the vtg synthesis in response to secondary exposure to E1 or E2. Compared to hepatocytes exposed only to MXCL or BPA, primary exposure to E2 increased the vtg synthesis in hepatocytes induced by MXCL or BPA by almost a factor of 2. Primary exposure to E1 increased vitellogenesis after secondary exposure to MXCL only marginally. The present results indicate that weakly estrogenic environmental chemicals such as MXCL and BPA can increase the sensitivity of carp hepatocytes towards endogenous estrogens with respect to VTG synthesis.

Key Words: carp hepatocytes; vitellogenin; in vitro; primary exposure; methoxychlor; bisphenol A.

Vitellogenin (vtg) has been used as a biomarker for exposure to xenobiotics with estrogenic properties in several in vivo and in vitro studies. Vtg is a precursor of the yolk proteins lipovitelline and phosvitin and is synthesized in the liver of female oviparous vertebrates as a consequence of estrogen-dependent gene expression. Estrogen receptor (ER) studies indicate that the ER is upregulated by (primary) exposure to xenoestrogens (Lazier and MacKay, 1993; Nimrol and Benson, 1997). Furthermore, in vivo experiments with rainbow trout indicate that pretreatment of animals with estrone (E1) increases the vitellogenic response of hepatocytes after a secondary exposure to estradiol (E2; van Bohemen et al., 1982). An enhanced inducibility of the vitellogenin gene via receptor induction is probably responsible for this increase in vitellogenesis. A persistent induction of the ER might be also responsible for the decrease of the lag time in E2 stimulated vitellogenesis in estrogen pretreated animals (“memory effect”), at least in some species as e.g., Xenopus (Lazier and MacKay, 1993). The vitellogenin gene is also present in male oviparous vertebrates but under natural circumstances endogenous estrogen levels are too low to induce vitellogenesis. However, if male fish are exposed to xenoestrogens, expression of the vitellogenin gene and vtg synthesis can occur (Callard and Ho, 1987).

There is serious concern that some persistent manmade chemicals found in the aquatic environment can disrupt endocrine development in wildlife and laboratory animals (Colborn et al., 1993; Guillette and Crain, 1995; Sonnenschein and Soto, 1998). Elevated vtg concentrations in the plasma of male trout, carp, and roach as well as an increased incidence of intersexuality in wild fish populations of roach have been associated with discharges from sewage treatment plants containing biologically active estrogenic compounds (Folmar et al., 1996; Jobling et al., 1998; Jobling and Sumpter, 1993; Purdom et al., 1994; Routledge et al., 1998; Rodgers-Gray et al., 2000).

In order to investigate the possible interactive effects of cocxposure in the environment we measured the effects of primary exposure of carp hepatocytes to xenoestrogens on vtg synthesis in response to secondary xenoestrogen exposure in the CARP-HEP assay (Smeets et al., 1999a).
The environmental contaminants methoxychlor (MXCL) and bisphenol A (BPA) were selected for this study because they can interact with the estrogen receptor but at concentrations that greatly exceed E2 or E1 concentrations (Eroschenko et al., 1996; Nimrod and Benson, 1997; Safe et al., 1998; Schlenk et al., 1998; Sheeler et al., 2000). MXCL and BPA have an estrogenic potency relative to E2 of respectively 1 $\times$ 10$^{-1}$ and 1 $\times$ 10$^{-4}$ in carp hepatocytes (Smeets et al., 1999b). The estrogenicity of these compounds has also been established in several other in vivo and in vitro studies using different methods, such as the E-screen and the Yeast Estrogen Screen, vitellogenin synthesis assays and competitive ER binding studies (Arnold et al., 1996; Christiansen et al., 2000; Chun and Gorski, 2000; Gray et al., 1989; Mehmood et al., 2000; Shelby et al., 1996; Sonnenschein and Soto, 1998).

MXCL is an organochlorine pesticide used as a substitute for DDT. In both fish and mammals MXCL has been classified as a proestrogen as biotransformation generates demethylated MXCL metabolites that elicit more estrogenic activity than the parent compound (Bulger et al., 1978). BPA is used in the manufacture of polycarbonate and epoxy resins. These resins can be found in various consumer products such as internal coatings for food cans, plastics, optical lenses, and restorative materials used in dentistry (Olea et al., 1996; Staples et al., 2000).

**MATERIALS AND METHODS**

**Experimental animals.** The genetically uniform strain of adult male carp (Cyprinus carpio) used in these experiments was obtained from the hatchery of the Fish Culture and Fisheries research group at Wagening Agricultural University. The fish were kept in Utrecht municipal tap water at a constant temperature of 24°C.

**Hepatocyte isolation and cell exposure.** Carp hepatocytes were isolated using a collagenase perfusion technique and cultured in phenol red-free DMEM/F12 medium (D2906, Sigma, St. Louis, MO), as described earlier (Smeets et al., 1999a). All in vitro experiments were performed twice, using isolated hepatocytes of 1 fish for each set of experiments. The concentrations of chemicals used for primary exposure (MXCL: 50 or 100 μM; BPA: 50 or 100 μM; E1: 1 or 10 nM; or E2: 0.1 or 1 nM) were chosen based on the criterion that these should induce vitellogenesis less than 10% of the response elicited by E2 (1000 nM). The concentration-response relationship for vtg induction by E2 in the CARP-HEP assay was determined earlier (Smeets et al., 1999a). All chemicals were dissolved in DMSO (final concentration 0.1%). Isolated hepatocytes from 1 fish (1 $\times$ 10$^7$ cells/ml medium) were put into single containers where either the stock solutions of the chemicals for primary exposure or only DMSO (0.1%; controls) were added. Then hepatocytes were seeded in 96-well tissue culture plates (180 μl/well; Greiner, Alphen a/d Rijn, The Netherlands). Each concentration of a compound was tested in 6 wells on at least 1 plate. Hepatocytes were pretreated with the xenoestrogens for a period of 48 h (Fig. 1). Cells were subsequently exposed (secondary exposure) to E1 (Sigma, St. Louis, MO), E2 (1 to 1000 nM; Sigma, St. Louis, MO), MXCL (Riedel de Hahn Ag, Seelze, Germany), or BPA (1 to 100 μM; Sigma, St. Louis, MO), or to DMSO alone, while refreshing the culture medium on the second and fifth day after isolation. Culture medium (containing the different concentrations of chemicals used for secondary exposure) was refreshed by removing 90% of the medium and replacing it carefully using a multichannel pipette. Hepatocytes were cultured for a total period of 8 days. At the end of the exposure period, culture medium was transferred to 96-well plates and kept at –70°C prior to vitellogenin analysis. The remaining cell monolayers were assessed for cell viability via the MTT assay.

**Cell viability and quantification of vitellogenin.** The viability of cells after secondary exposure (on day 8) was determined by measuring the mitochondrial dehydrogenase activity using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) as substrate (Denizot and Lang, 1986). Cell viability was also assessed by microscopic examination of the cell morphology and the state of cell-to-cell contacts on days 1, 2, and 5 of the assay. A competitive indirect ELISA was used to determine the level of vtg in the culture medium on day 8 as described earlier (Smeets et al., 1999a). Plasma of an E2 treated female carp, containing approximately 45 ng/ml vtg was used for the standard curve dilution. The detection limit of the ELISA was defined as the vtg level corresponding to the lower limit of the linear part of the standard curve. Depending on the sensitivity of the hepatocytes the detection limit of the ELISA corresponded with an exposure of the cells to between 2 and 5 nM E2 without pretreatment.

**Statistical analysis.** Statistical calculations were performed using the computer program SPSS. Statistical significance ($p < 0.01$) among the treatments was calculated using a one-way ANOVA and a post hoc test (least squares difference) with Bonferroni correction.

**RESULTS**

**Cell Viability**

Cell viability was determined by comparing mitochondrial MTT of hepatocytes exposed to medium containing DMSO (controls) or to medium containing the chemicals (dissolved in DMSO). Compared to the controls up to 70% greater MTT activity was observed after primary exposure of hepatocytes to BPA (50 or 100 μM) and secondary exposure to 1 or 10 nM E2. Secondary exposure of hepatocytes to 50 or 100 μM MXCL resulted in MTT activity reduction that was approximately 30% less than the mitochondrial activity of the controls.

Microscopic observation revealed that during the first day after seeding carp hepatocytes were similar in shape to freshly isolated cells, but with a limited degree of intercellular contact. Two days after seeding the hepatocytes formed clusters with a high degree of intercellular contact. After 5 days in culture the degree of cluster formation was similar to the monolayer hepatocyte cultures of rainbow trout (Flouriot et al., 1993). All cell layers remained attached to the bottom of the plates throughout the experiment.

**Primary Exposure to Xenoestrogens**

Hepatocytes that were pretreated on day 1 with several xenoestrogens showed increased sensitivity for vtg induction.
by secondary exposure to xenoestrogens (Table 1). Compared to cells exposed only to E2 or E1, primary exposure to MXCL (50 or 100 \( \mu \text{M} \)) increased the vtg production in response to 1000 nM E2 or E1 by 4- and 2-fold, respectively (Figs. 2 and 3). The enhanced response to E1/E2 exposure was similar whether 50 or 100 \( \mu \text{M} \) MXCL was used for primary exposure. Compared to cells without pretreatment primary exposure to 50 or 100 \( \mu \text{M} \) BPA, with subsequent exposure to E2 (1000 nM), increased the production of vtg by approximately 5- to 7-fold (Fig. 2). In the case of BPA pretreatment (50 or 100 \( \mu \text{M} \)), secondary exposure to 1000 nM E1 resulted in 4 and 5 times greater vtg induction than without primary exposure (Fig. 3). Compared with cells not pretreated, primary exposure to E1 (1 or 10 nM) or E2 (0.1 or 1 nM) followed by secondary exposure to 1000 nM E1 or E2 did not affect vitellogenesis (data not shown). As a consequence of pretreatment with 1 nM E2 or 10 nM E1, vtg synthesis in hepatocytes induced by 100 \( \mu \text{M} \) MXCL increased by a factor of 2.3 and 1.6, respectively (Fig. 4). Vitellogenesis doubled after primary exposure of the hepatocytes to 1 nM E2 and secondary exposure to BPA 100 \( \mu \text{M} \) (Fig. 5). No influence on vitellogenesis was observed comparing cells exposed to 100 \( \mu \text{M} \) BPA with cells pretreated with 1 or 10 nM E1 and subsequent exposure to BPA (100 \( \mu \text{M} \); Fig. 5).

### DISCUSSION

Changes in the synthesis of vtg were observed in the hepatocytes of individual carps after exposure to xenoestrogens, rather caused by a compound- and concentration-dependent effect than by cytotoxic effect. MTT activity remained unchanged for most of the compounds tested. Only exposure to 50 or 100 \( \mu \text{M} \) MXCL reduced MTT activity. However, exposure to both concentrations of MXCL still increased vitello-

### TABLE 1

Summary of the Results of the Experiments with and without Primary Exposure to Xenoestrogens in Carp Hepatocytes

<table>
<thead>
<tr>
<th>Primary exposure</th>
<th>Secondary exposure</th>
<th>Effect on vitellogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (1 nM)</td>
<td>Estradiol (1000 nM)</td>
<td>100</td>
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<tr>
<td>Estradiol (1 nM)</td>
<td>Estrone (1000 nM)</td>
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<td>Estrone (10 nM)</td>
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<td>Estrone (10 nM)</td>
<td>Estrone (1000 nM)</td>
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<tr>
<td>Estradiol (0.1 nM)</td>
<td>MXCL (100 ( \mu \text{M} ))</td>
<td>3</td>
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<td>Estradiol (1 nM)</td>
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<td>Estrone (1 nM)</td>
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<td>Estrone (10 nM)</td>
<td>MXCL (100 ( \mu \text{M} ))</td>
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<td>Estradiol (0.1 nM)</td>
<td>BPA (100 ( \mu \text{M} ))</td>
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<td>BPA (100 ( \mu \text{M} ))</td>
<td>Estradiol (1000 nM)</td>
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*Note. Vtg is expressed as a percentage of the induction by 1000 nM estradiol (without pretreatment).

*Values significantly different from their controls (without primary exposure; \( p < 0.01 \)).
genesis, which would not be expected in the case of cytotoxicity of MXCL. The reduction of MTT activity of cells exposed to 50 or 100 μM MXCL may be a function of a shift in energy balance within the cells. Future experiments are necessary to substantiate this suggestion. A decrease of MTT activity in combination with an increase of vitellogenesis has been described earlier in the CARP-HEP assay by Smeets et al. (1999a). Further investigation will also be necessary to explain the mechanisms behind the increase of MTT activity after pretreatment of hepatocytes with BPA (50 or 100 μM) and secondary exposure to 1 or 10 nM E2. A comprehensive microscopic examination of the hepatocytes, which was used as an additional parameter for cell viability, did not reveal any signs of cell decay, and the cell layers remained attached to the bottom of the wells throughout the experiment (which is also a sign of viability of the cells). Therefore, it can be concluded that the added compounds did not influence the viability of the cells.

From a quantitative point of view, changes in the synthesis of vtg were observed in the hepatocytes of individual carps after exposure to xenoestrogens. These quantitative differences in vitellogenesis might be due, in part, to individual differences in sensitivity to estrogens. Also the age of the fish and seasonal variations in sensitivity to estrogens might play a role. Thus we normalized the results of each experiment to the level of vtg induced by 1000 nM E2 (without primary exposure) and expressed vtg synthesis as a percentage of this level for comparison purposes. However, when normalizing the results of the different sets of experiments, we obtained comparable (qualitative) results in the dose response relationships exposing the hepatocytes to xenoestrogens.

In spite of the fact that they have only little estrogenic potency, MXCL and BPA are able to increase vitellogenin synthesis significantly when combined with a subsequent exposure to E2 or E1. Vitellogenesis was doubled after primary exposure to E2 followed by exposure to 100 μM MXCL or BPA. Primary exposure to E1 almost doubled the synthesis of vitellogenin after secondary exposure to MXCL (100μM). However, no enhancement of vitellogenesis by secondary exposure to BPA was observed after primary exposure to E1. A possible explanation for the observed enhancement of vitellogenesis could be that pretreatment with the xenoestrogens at a low dose level could induce the ER population (priming) at a concentration below the threshold for vitellogenin synthesis in males. Consequently, subsequent exposure to another xenoestrogen would induce vitellogenesis to a greater level than without primary exposure, due to upregulation of the ER. This hypothesis is supported by the findings from in vivo studies that primary exposure to estradiol or to the xenoestrogen nonylphenol increased the number of estrogen binding sites. For example, repeated exposure of catfish (on days 1, 4, and 7) to 0.6 mg/kg E2 or to 60.5 mg/kg nonylphenol increased the number of ER in the cytosolic fraction compared to control males (Nimrod and Benson, 1997). Another example for the upregulation of the ER is the increased capacity of the uterus of ovarioectomized rats to bind E2 after E2 pretreatment for 3 days (2 μg/100g) and subsequent exposure to E2 (2 μg/100g; Eisenfeld and Axelrod, 1966).

Since primary exposure to the endogenous estrogens followed by exposure to E1 or E2 failed to increase vitellogenesis, factors other than upregulation of ER may affect vitellogenesis if only endogenous estrogens are involved. There may be different E2 receptor populations involved (with higher and lower affinity for endogenous estrogens). It has been reported that an ER population with a lower affinity for endogenous estrogens increased when channel catfish were pretreated with E2 (Nimrod and Benson, 1997). Changes in affinity for endogenous estrogens have also been reported for hepatic ER populations of brown and rainbow trout (Campbell et al., 1994). It would appear that an increase in the number of hepatic ER in

FIG. 4. Effects of E1 or E2 pretreatment on vitellogenesis after secondary exposure to MXCL in carp hepatocytes. Error bars represent SD of 6 determinations, except for controls (n = 12). *Values significantly different from their controls (without primary exposure; p < 0.01). Controls were exposed to an equivalent concentration of DMSO only.

FIG. 5. Effects of E1 or E2 pretreatment on vitellogenesis after secondary exposure to BPA in carp hepatocytes. Error bars represent SD of 6 determinations, except for controls (n = 12). *Values significantly different from their controls (without primary exposure; p < 0.01). Controls were exposed to an equivalent concentration of DMSO only.
response to primary exposure to E1 and E2 is compensated by a decrease in affinity to those estrogens. This could partly explain the lack of enhancement of vitellogenesis observed by primary and secondary exposure to endogenous estrogens in the carp hepatocytes. Further studies, including receptor binding kinetics and affinities in these carp hepatocytes might elucidate the mechanisms behind these effects.

The present and previous results obtained using the CARP-HEP assay (Letcher et al., submitted; Smeets et al., 1999b) demonstrate that BPA has a lower estrogenic potency than MXCL. However primary exposure to BPA is more effective in enhancing the vitellogenin production in response to E2 or E1 than pretreatment with MXCL. A possible explanation for these differences could be that while exposing hepatocytes for period of 7 days to MXCL, not only the parent compound, but also its bioactivated metabolites, bind to the ER. It has been reported that the o-demethylated metabolites of MXCL have a greater binding affinity to the hepatic ER of channel catfish and the rat uterine estrogen receptor than their parent compound (Nimrod and Benson, 1997; Ousterhout et al., 1981; Schlenk et al., 1998). Therefore the bioactivated MXCL compounds probably induce vitellogenesis more than the parent compound does. Probably the pretreatment period of 48 h is not long enough to bioactivate MXCL as effectively as after 6 days of secondary exposure.

In vivo experiments with rainbow trout showed that repeated pretreatment of the animals with E1 increased the vitellogenic response to estradiol treatment (van Bohemen et al., 1982). This is in contrast to our in vitro results obtained with carp hepatocytes. A possible reason for this difference may be that the in vivo effects we described on vitellogenesis in the trout experiments are more evident due to the greater frequency of exposure. Both the pretreatment with E1 and the secondary exposure to E2 were repeated daily for 7 days.

These results indicate that relatively high concentrations of environmental pollutants with a weak estrogenic potency, such as MXCL and BPA, can increase the sensitivity of vitellogenesis observed by primary and secondary exposure to endogenous estrogens in the carp hepatocytes. Further studies, including receptor binding kinetics and affinities in these carp hepatocytes might elucidate the mechanisms behind these effects.

REFERENCES


