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Evaluation of Environmental Estrogens with a Fish Cell Line

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Estrogenic chemicals are released to the aquatic environment from multiple sources of pollution. Indeed, municipal effluents are known to contain surfactants such as polyethoxylates and their biodegradation products, as well as metabolites of estrogens (Gillesby and Zacharewski 1998). These chemicals are well known to bind the estrogen receptor and initiate the synthesis of vitellogenins (Vg) in oviparous organisms (Flouriot et al. 1995). Vg are glycosylated lipoprotein that is produced in the liver of oviparous vertebrates and transported by the blood into the oocytes for energy reserves. Whereas the stimulation of vitellogenesis in females or males can be deleterious because of the draining of their energy reserves, lower Vg levels in the developing embryo can also be harmful since the hatchling would have a lower energy reserves. Vg can be measured by a variety of assays, including the highly specific immunoassays, the DNA probe hybridization for mRNA determination, and the alkali-labile phosphates assay (Ren et al. 1996, Mourot and LeBail 1995, Copeland et al. 1986).

The presence of estrogens in the environment can be assessed through both *in vivo* and *in vitro* bioassays (Gillesby and Zacharewski 1998). Because *in vivo* bioassays are relatively costly and require the sacrifice of organisms, *in vitro* bioassays have been widely adopted to screen for the presence of estrogenic compounds in environmental samples. The biosynthesis of Vg in primary cultures of rainbow trout hepatocytes has been proposed as an *in vitro* fish bioassay for the detection of estrogenic chemicals in the aquatic environment (Maitre et al. 1986). Indeed, the evaluation of Vg in rainbow trout hepatocytes has been used successfully as a microscale bioassay for detecting estrogenic properties in both single compounds and complex mixtures (Pelissero et al. 1993, Ren et al. 1996). However, rainbow trout hepatocytes still require the sacrifice of trouts, albeit far fewer than when using whole organisms, since primary cultures of hepatocytes are viable for only seven days in culture (Klauning et al. 1985). In this respect, a readily-available continuous fish cell line that would respond to estrogens is lacking. Rainbow trout hepatoma cells (i.e. from the RTH-149 cell line) are likely to produce Vg upon exposure to estrogen because the transformed cells originated from liver epithelial cells (Lorenzen and Okey 1990). The purpose of the study was therefore to verify

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whether this transformed cell line produced Vg after exposure to estrogens and municipal effluents. Moreover, the sensitivity of the hepatoma cell line was compared with that of primary cultures of rainbow trout hepatocytes.

MATERIALS AND METHODS

Rainbow trouts (*Oncorhynchus mykiss*) measuring 10–15 cm long were obtained from a commercial fish hatchery and fed trout chow once daily. Hepatocytes were collected using the double perfusion method (Klauning et al. 1985). Cells were distributed in a 48-well microplate at a density of 1 x 10^6/mL in sterile L-15 medium supplemented with 1% fetal bovine serum (FBS), 1000 units of penicillin, 10 mg/L of streptomycin, and 25 μg/L of amphotericin B. The livers of three fish were pooled for each experiment. Rainbow trout hepatoma cells were purchased from American Cell Culture Type Collection (USA). Immediately after reception, the cells were thawed at room temperature, and diluted 1:10 in L-15 media containing 7.5% FBS and antibiotics. They were centrifuged at 500 x g for 5 min, plated in 10 mL of L-15 media containing 7 % FBS, and grown at 15°C in a humidified incubator.

The cells were exposed to β-estradiol (range 0.1–10 μM) and municipal wastewaters (1–50% v/v). The hepatocytes were plated in 48-well microplates at 1 x 10^6 cells/mL while hepatoma cells were plated at 5 x 10^4 cells/mL in 96-well microplates. The microplates were incubated for 48 h at 15°C in a humidified incubator.

Cell viability was determined by the neutral red uptake assay (Dierickx and Van deVyver, 1991). The decrease of neutral red uptake by cells is directly related to the loss of cell viability. Briefly, 5 x 10^4 cells were mixed with 0.004% neutral red for 90 min at room temperature. The cells were centrifuged at 500 x g for 5 min, and the incubation media carefully removed. The cells were solubilized in 50% methanol with 2% glacial acetic acid for 15 min before reading at 540 nm in a microplate reader.

The amount of Vg secreted into the extracellular medium was evaluated using the organic alkali-labile phosphate method (Gagné and Blaise 1998). Briefly, 200–750 μL of the extracellular medium was extracted with 500 μL tert-butyl ether for 30 min at room temperature. A volume of 325 μL was collected and mixed with 100 μL of NaOH 2 M for 60 min to release the labile phosphates present in the ether phase. The emulsion was centrifuged (10 000 x g, 2 min) and the level of total phosphate in the aqueous phase was quantified using the phosphomolybdenum method (Stanton 1968).

The Vg mRNA levels were measured by means of a chemoluminescent in situ hybridization assay (CISH) using PCR-generated DNA probes labelled with digoxigenin (Gagné and Blaise 1998). Briefly, a digoxigenin-labelled DNA probe

was produced by asymmetric polymerase chain reaction with primers selected to amplify the exon 30 region at nucleotides 386 to 586 of trout Vg gene (Mouchel et al. 1996). The primers were 5'-TTA-AAT-GTA-GCA-TGG-TCA-3 (template strand) and 5'-ATG-TCA-GAG-ATT-TTC-ACA-3' (complementary strand). The PCR products were analysed by 2% agarose gel electrophoresis to confirm amplification of an approximately 200 base pair DNA. Following exposure to estradiol and the wastewaters, the cells were washed in PBS (resuspended in PBS and centrifuged at 100 x g for 2 min) and resuspended for 10 min in 150 µL of 3.7% formaldehyde in PBS containing 5 mM $MgCl_2$. The fixative was removed and the cells were treated with 0.2% Tween-20 and 0.5% bovine serum albumin (BSA) in PBS for 15 min. The cells were then hybridized with 2 µg of DIG-DNA probe in SSC 4X (SSC 4X: 600 mM NaCl and 60 mM citrate, pH 7.2), containing 0.1% each of SDS, polyvinylpyrolidone, Ficoll and BSA. The cell suspension was heated at 90°C for 2 min then at 58°C for 1 hour to allow hybridization. After this incubation period, the cells were washed three times in PBS and exposed to a DIG secondary antibody linked to alkaline phosphatase (Boehringer Mannheim) in PBS containing 3 % BSA for 45 min at 37°C. The cells were washed four times in PBS and the alkaline phosphatase activity in the cells was measured using (3, 4-methoxyspiro [1,2 dioxethane-3,2'(5'-chloro)-tricyclo decan]-4 yl)phenyl phosphate as the substrate, which yields luminescence (Dynatech Laboratories, ML-100) upon dephosphorylation by the enzyme (Boehringer Mannheim). Alkaline phosphatase activity was corrected for cell density by total DNA measurement using Hoescht dye (West et al. 1985).

The cells obtained from each preparation were exposed to the test samples in four replicates ($n = 4$). Cell viability, EROD activity, and extracellular Vg data were subjected to an analysis of variance where critical differences between exposed and unexposed groups were determined with Dunnett's t test. The lowest observable effect concentration (LOEC) in % v/v, and the no observable effect concentration (NOEC) in % v/v were then determined. A toxicity threshold (TT) was calculated as follows: $TT = [NOEC \times LOEC]^{1/2}$. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The exposure of rainbow trout hepatoma to estradiol-17β induced the levels of organic ALP in the extracellular medium and Vg mRNA levels (Figure 1). Hepatocytes responded similarly to estradiol-17β (Figure 2). Indeed, the exposure of trout hepatocytes to estrogens starts the synthesis of Vg (Maitre et al. 1986, Pelissero et al. 1993). It is noteworthy that ALP levels in untreated hepatoma cells where lower than those found in unexposed hepatocytes which suggest that isolated hepatocytes have more baseline levels of phosphorylated lipoproteins, including Vg, than hepatoma cells. The lower baseline value of ALP in hepatoma cells confers increased sensitivity to detect environmental estrogens. Our study is the first to show that the exposure of trout hepatoma cells to estradiol increases the

levels of Vg mRNA in cells and the levels of Vg in the cell culture medium. These results suggest that hepatoma cells, which are thought to originate from liver parenchymental cells, still possess a functional and inducible Vg gene. It appears that hepatoma cells retain many hepatocyte characteristics such as the presence of Ah receptor (Lorenzen and Okey 1990) and metallothionein (MT) induction capacity (Olsson et al. 1990). However, hepatoma cells induced significantly less MT than did primary cultures of trout hepatocytes, suggesting that a difference in the intensity of gene expression can be expected. According to our results, hepatoma cells and hepatocytes had increased mRNA levels of 1.3- and 2.75-fold, respectively. The levels of extracellular Vg, as determined by the organic ALP assay, increased 3.5- and 1.5-fold for hepatoma cells and hepatocytes, respectively. These results suggest that hepatoma cells are more efficient at producing and excreting Vg than are hepatocytes, while the opposite is true for the production of Vg mRNA. It has been shown that the half-life of Vg mRNA can be modulated upon exposure to estrogens (Flouriot et al. 1995, Gagné and Blaise 1998), in that lower correspondence can occur between the levels of Vg proteins and mRNA levels.

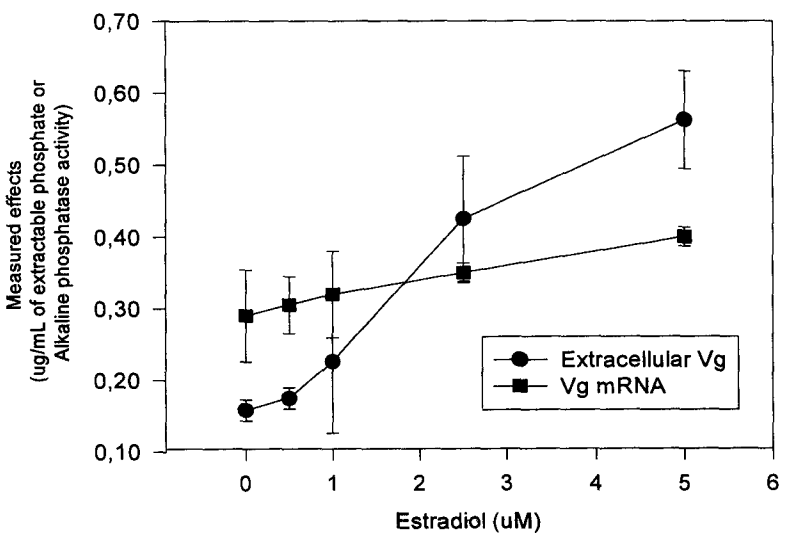

Figure 1. Exposure of rainbow trout hepatoma cells to estradiol.
Rainbow trout hepatocytes were exposed to several concentrations of estradiol for 48 h at 15°C. Mean ± SD, $n = 4$.

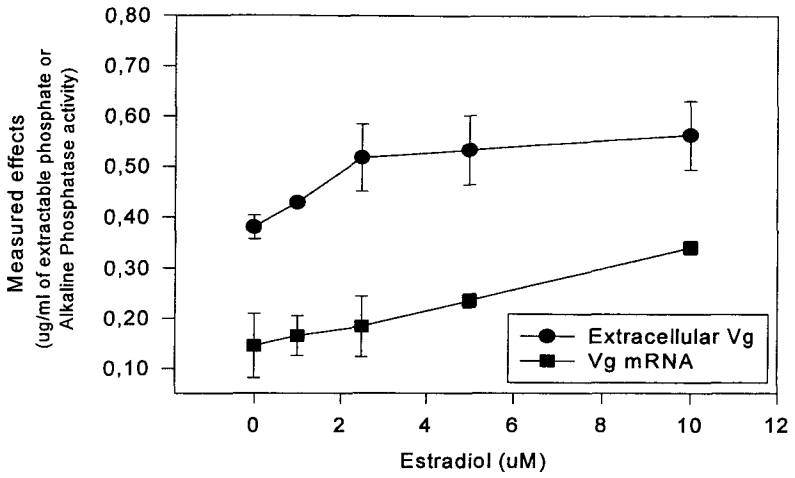

Figure 2. Exposure of rainbow trout hepatocytes to estradiol.
Rainbow trout hepatocytes were exposed to several concentrations of estradiol for 48 h at 15°C. Mean ± SD, $n = 4$.

The estrogenic properties of municipal wastewaters were also examined in both rainbow trout hepatocytes and hepatoma cells (Table 1). Municipal wastewaters have been shown to be estrogenic most of the time in trout hepatocytes with the Vg assay (Gagné and Blaise 1998). Our results indicate that both cell systems are concordant in detecting the presence of estrogens in these wastewaters ($R = 0.99$, $p < 0.001$). The hepatoma cells appear to be more sensitive to detecting estrogenic activity than are trout hepatocytes. However, this difference in sensitivity can be explained, at least in part, by the different cell densities used during the exposure period. Indeed, the cell density of the hepatoma cells was 20 times lower than that of the hepatocytes.

In conclusion, rainbow trout hepatoma cells produce Vg in response to estradiol and municipal wastewaters. Moreover, the results also show that the Vg induction threshold values are highly correlated with the results obtained with primary cultures of rainbow trout hepatocytes. However, the relative induction factor for Vg mRNA and extracellular organic ALP differs between cell systems, suggesting differences in gene expression at both the transcriptional and translational levels. The secretion of Vg in extracellular media may also differ between cell models. The results suggest that this transformed fish cell line is a promising tool to screen for environmental estrogens. However, the specificity of Vg synthesis by other xenobiotics has yet to be determined in hepatoma cells.

Table 1. Estrogenic properties of municipal effluents in trout hepatocytes and hepatoma cells.

Effluent	Relative population size	LOEC (%)[a]	
		Hepatocytes (1×10^6/mL)[b]	Hepatoma (5×10^4/mL)[b]
1	1	10	1
2	1	25	2
3	1	50	ND
5	0.025	25	2
6	0.0125	10	1

a: The Vg mRNA data are reported in LOEC in % v/v.
b: The relative cell densities are reported.
ND: Not determined.

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