Bull. Environ. Contam. Toxicol. (1999) 62:723-730

Bulletin of Environmental Contamination and Toxicology

Estrogenic Effects of Organic Environmental Extracts with the Trout Hepatocyte Vitellogenin Assay

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Received: 10 January 1999/Accepted: 24 March 1999

An increasing number of chemicals is found in the environment that can modulate or perturb the endocrine system. Important physiological systems under endocrine control such as growth, sexual differentiation, reproduction and metabolism are likely to be altered by these chemicals. For example, feminization in fish or alligators, hermaphrodism, imposex on gastropods and increased female/male ratios in turtles are the result of endocrine disruption (Gillesby and Zacharewski 1998). Xenoestrogens represent the most characterized class of endocrine disrupters. Chemicals like nonylphenol, lindane, bisphenol-A were identified to possess some capacity to influence the estrogen receptor pathway. For example, those chemicals bind to the estrogen receptor and start the synthesis of vitellogenin (Vg) in both male and female fishes or other oviparous organisms (Flouriot et al. 1995). Vg is a lipophosphoprotein that is produced in the liver of oviparous vertebrates under estrogenic stimulation and transported by the blood into the oocytes for energy reserves. Whereas stimulation of vitellogenesis in females or males can be deleterious because of the draining in their energy reserves, lower Vg levels in the developing embryo could be deleterious since the hatchlings would have less energy reserve. Vg biosynthesis in rainbow trout hepatocytes is stimulated by estrogens and was proposed as an *in vitro* bioassay to detect estrogenic chemicals in the aquatic environment (Maitre et al. 1986; Pelissero et al. 1993; Ren et al. 1996). Vg production by trout hepatocytes can be used to screen chemicals in the activation of the Vg gene which is under control of the estrogen receptor.

In the environment, both estrogen antagonists and agonists are present. Indeed, heavy metals such as cadmium (Cd) are known to decrease Vg mRNA and protein synthesis in trout hepatocytes (Olsson et al. 1995). In addition, polycyclic aromatic hydrocarbons are a class of chemicals that are also anti-estrogenic since they can decrease Vg gene expression (Anderson et al. 1996; Monosson et al. 1994). It was found that antagonist effect of PAHs on vitellogenesis takes place by the Ah-receptor in the induction of cytochrome P4501A1 enzyme activities - hence the term Ah-receptor mediated inhibition of Vg gene expression (Anderson et al. 1996). Therefore, the interplay between the antagonists and agonists is likely to

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determine the outcome of the estrogenic properties of the mixture. In the attempt to eliminate these interfering factors, dichloromethane extraction of environmental samples should eliminate the presence of ionic metals. However, PAHs would be included in the final extract, thus interfering again in the detection of estrogenic chemicals. We can propose the hypothesis that since PAHs (or chemicals interacting with the Ah-receptor) are less polar that estradiol-17β, chemicals sharing the same polarity of estradiol-17β would be more estrogenic than those sharing similar polarity of PAHs. The purpose of this study was to evaluate the effects of removing non-polar chemicals from the polar chemicals in the extracts on the resulting estrogenic potential. In this study, numerous samples such as rain or snowfalls, industrial effluents, marine and freshwater sediments and municipal suspended matter were screened for the presence of estrogenic properties.

MATERIALS AND METHODS

All extractions were performed in analytical grade dichloromethane (DCM). In the case of liquid samples (e.g., industrial effluents or rainfalls), liquid-liquid extractions were performed. Briefly, the liquid samples were filtered through a 0.45 µm membranes and one volume of dichloromethane was added to the liquid sample and mixed for 1 hr at room temperature. Afterwards, DCM extracts were passed through a sodium thiosulfate column to remove trace amounts of water before drying under nitrogen stream. The material was resuspended in 0.1 mL of DCM. An operational blank was included during all steps that contained DCM and bidistilled water only. In the case of solid samples (e.g., sediments, suspended matter), extraction was achieved using the Soxhlet procedure (Hunchak and Suffet, 1987) for 8 h. The extracts were evaporated under nitrogen stream and resuspended in 0.1 mL of DCM. Polarity fractionation of the extracts was achieved according to normal phase chromatography procedure (Grifoll et al. 1990). Normal phase mini-extraction columns (2,3-dihydroxypropoxypropyl-silica, International sorbent technology, USA) were equilibrated in hexane-10% DCM. The DCM extracts were diluted 1/10 in hexane and adsorbed to the column. The non-polar fraction was obtained by collecting the eluate (non-absorbed material) and washing with 1 mL hexane followed with 1 mL of hexane/DCM (1:1). The polar fraction was obtained by eluting twice with 1 mL methanol-DCM (1:1). The (non-)polar fractions were evaporated to dryness under a nitrogen stream and resuspended in 100 µL of dimethylsulfoxide (DMSO) for the hepatocyte assays. An operational DCM blank was included and the column's performance was verified with standard amounts estradiol-17β and benzo(a)pyrene (BaP).

Rainbow trout (*Oncorhynchus mykiss*), 10-15 cm long, were obtained from a commercial fish hatchery and fed trout chow once daily. Hepatocytes were collected by 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 (at 320 mOsmol/kg H_2O) supplemented with 2% fetal bovine serum, 1000 units of penicillin, 10 mg/L of streptomycin and 25 µg/I. of amphotericin B. The cells were exposed to 0.0016, 0.008, 0.04, 0.2 and 1% v/v of the extracts or to concentrations of β-estradiol (ranging from 0.1 to 10 µM). The microplate was incubated for 48 h at 15°C in a humidified incubator. The livers from three fish were pooled for each experiment.

Cell viability was determined by the propidium iodide (PI) exclusion test (Zucker et al. 1988). Briefly, 50 x 10^3 cells were mixed with 25 µg/mL of PI in phosphate buffered saline (PBS) for 30 min. The cell suspension was then analyzed using a microplate fluorescence plate reader, with dead cells becoming fluorescent at 600 nm upon excitation at 485 nm. The proportion of viable cells (exposed *versus* unexposed) was determined with 10% DMSO, a positive control for cell death that permeabilizes cell membranes. Cell fluorescence was corrected with cell density (fluorescence of PI-DNA in wells/absorbance at 600 nm) before addition of the PI dye.

The amount of vitellogenin (Vg) secreted into the extracellular medium was evaluated by the alkali-labile phosphate (ALP) method (Gagné and Blaise 1998 ; Pereirra et al. 1992).

Cytochrome P4501A1 activity was measured by following deethylation of 7-ethoxyresorufin (Hahn et al. 1996) with some modifications. Briefly, 1 x 10^5 cells were added to 10 µM of 7-ethoxyresorufin in PBS containing 0.015% triton X-100 for 30 min at 22°C. Afterwards, fluorescence was measured at 590 nm upon excitation at 540 nm. Fluorescence intensity was corrected for blank values (t = 0 min) and cell density as measured at 600 nm. Standards of 7-hydroxyresorufin were used to calculate the amount of product formed over time.

Cells obtained from the same 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: $TT = [NOEC \times LOEC]^{1/2}$. The TT data were corrected by the weight of each of the samples tested. Significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Exposure of rainbow trout hepatocytes to estradiol-17β induced the levels of ALP in the extracellular medium (Figure 1). Moreover, the relative levels of ALP were found to be significantly reduced when benzo(a)pyrene (BaP) was present corroborating the observation that Ah-receptor activation reduces Vg gene expression (Anderson et al. 1996).

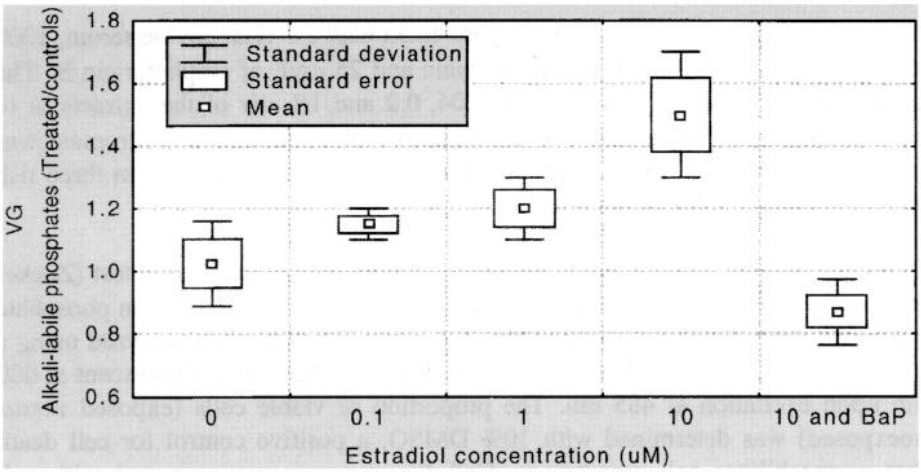

Figure 1. Elevation of Vg levels in hepatocytes exposed to estradiol-17β.
Cells were exposed to different concentrations of estradiol for 48 h at 15°C. Afterwards, the levels of ALP in the extracellular media were determined. In one case, cells were co-exposed to 125 µM BaP and 10µM estradiol. The data represent the results from n=4 experiments.

Organic extracts can be fractionated according to the polarity of the molecules by normal phase chromatography. Indeed, it was found that BaP eluted in the non-polar fraction when DCM extract of sediments was spiked with BaP (Figure 2). Estradiol-17β, which is more polar that BaP, forms hydrogen bonds to the Si-OH matrix which requires elution with a more polar solvent (DCM/methanol). We obtained the same results with a mixture containing only BaP and estradiol-17β. Normal phase chromatography of organic extracts of sediments elutes most of the PAHs after elution at 20% DCM in hexane while the more polar chemicals like coprostanol elutes only in 75% DCM in hexane while nonylphenol and cholesterol elutes in 50% methanol in DCM. Amino compounds and carboxylic acids would remain bound to the column since they would require elution by a more polar solvent such diethyl ether (Grifoll et al. 1990).

The effects of the DCM extracts along with the non-polar and polar fractions on rainbow trout hepatocytes were determined (Table 1). The extracts proved to be toxic in the original extracts in municipal suspended matter and in both freshwater and marine sediments. No toxicity was observed in the industrial effluents, the snow samples and the reference sediments. We found that the most toxic samples were from the municipal suspended solids and the freshwater sediment highly contaminated with PAHs and organochlorinated pesticides (Balch et al. 1995). The highest EROD activity value, which is induced by coplanar aromatic hydrocarbons in general, was induced in the marine sediment extract obtained from a commercial harbour. It was also induced in PAH-contaminated sediments and municipal suspended matter. It is noteworthy to note that population size of the cities seems to have no influence on the potency to induce EROD. The non-polar fractions of these samples proved to contain most of the EROD-inducing capability. Interestingly some EROD-inducing activity is obtained in the polar

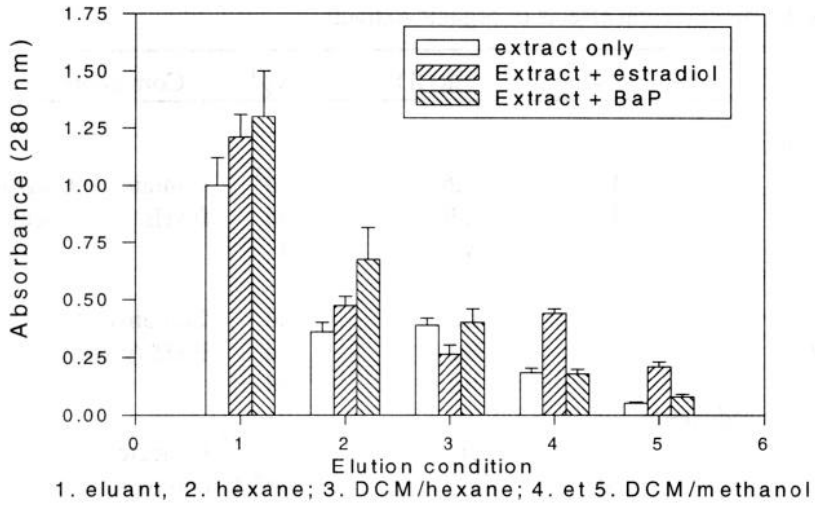

Figure 2. Normal phase fractionation of organic environmental extracts.
The mini column contained 100 µg of the solid phase (Si-OH) equilibrated with 90% hexane-10% DCM. BaP and estradiol-17β (10 µM of each) were added separately to a sediment extract. The non-polar fraction corresponds to the material obtained with elution steps 1, 2, and 3 while the polar fraction corresponds to elution steps 4 and 5. The data represents the results from n=3 experiments.

fractions suggesting either that some oxidized PAHs still possess EROD-inducing activity or that the material retained on the matrix competes with the solvent to retain PAHs. The last possibility is consistent with the experimental observation that these two sediment extracts were very dense and may have been saturated by organic material. Almost all extracts had the potential to induce Vg in rainbow trout hepatocytes with the exception of the clean freshwater sediment extract. Snow extracts were estrogenic while the one taken at the curb was less estrogenic and induced EROD activity. In conclusion, estrogenic activities were mainly observed in the polar fractions and the extracts. In some case, some extracts proved to be less or not estrogenic at all while the polar fraction proved to be estrogenic when the non-polar fraction was removed. This result suggests that some extracts may be classified not estrogenic while, in fact, they possess estrogenic chemicals.

Acknowledgments. We thank the St. Lawrence Center for supporting this research work.

Table 1. Biochemical effects of organic extracts.

Samples	Viability[1]	ERODV g[1]		Comments
1. Freshwater sediments				
E[2]	4	5.6	nd[3]	Contains 100 µg of
NP[2]	3.3	4.9	nd	PAHs/g sediment
P[2]	4	4	4	
E	nd	nd	nd	Contains 0.9 µg of
NP	nd	nd	nd	PAHs/g sediment
P	nd	nd	nd	
2. Marine sediments[2]				
E	3.1	6.1	(-)3.1[4]	Collected from a
NP	nd	5.1	(-)3.1	commercial
P	nd	nd	nd	harbour
E	nd	nd	3.4	Collected from at 5
NP	nd	nd	nd	km from the
P	nd	nd	3.4	harbour
3. Municipal suspended matter[3]				
E	4.1	4.8	5.7	Population size :
NP	4.1	4.7	nd	2×10^6
P	nd	nd	5.7	
E	4.2	4.9	4.2	Population size:
NP	3.5	4.2	3.5	1×10^4
P	4.2	3.5	4.9	
4. Industrial[4]				
E	nd	0.14	nd	Final effluent from
NP	nd	0.14	(-)0.14	a fuel-producing
P	nd	nd	nd	plant
E	nd	4.51	5.2	Involved in paper
NP	nd	nd	nd	recycling
P	nd	nd	5.18	
5. Snow				
E	nd	nd	1.33	Snowfall
E	0.64	0.64	0.64	Street snow

1. Data are expressed in log of toxicity units (TU) /g of dry solid (liquid) weight. 2. E : DCM extract ; NP : non-polar fraction ; P : polar fraction. 3. nd : no effect detected at the highest concentration tested (1%). 4. the term (-) signify a decrease of effect or activity while the sign - alone indicates the actual value.

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