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THE *IN VIVO* EFFECT OF COMBINATIONS OF OCTYLPHENOL, BUTYLBENZYLPHTHALATE AND ESTRADIOL ON LIVER ESTRADIOL RECEPTOR MODULATION AND INDUCTION OF ZONA RADIATA PROTEINS IN RAINBOW TROUT: NO EVIDENCE OF SYNERGY

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Abstract

Juvenile rainbow trout were given intraperitoneal (IP) injections of (i) octylphenol (OP) and butylbenzylphthalate (BBP) or (ii) octylphenol and estradiol (E). Both pairs of compounds were administered singly and in combination, at two different dose levels. Modulation of the hepatic estradiol receptor (ER) binding capacity and induction of hepatic zona radiata proteins (Zrp) were utilised as indicators of estrogenic effect. In treatment (i) OP caused a significant (~2-fold) upregulation of the ER and a significant (~3-5 fold) increase in Zrp at both doses compared to control, vehicle-injected fish. In contrast, no effect on the ER was observed in BBP-treated fish, but plasma Zrp levels were significantly reduced. No synergistic effect was observed on ER modulation or Zrp induction in fish receiving OP and BBP in combination. In treatment (ii) both OP and E caused a significant (~2-fold) upregulation of the ER and a significant (~6-7 fold) increase in plasma Zrp at both dose levels compared to controls. The effect of OP and E in combination, on both ER modulation and induction of Zrp, was additive and not synergistic.

Key words: rainbow trout, estradiol receptor, estradiol, environmental estrogens, octylphenol, butylbenzylphtalate, zona radiata proteins, synergism.

INTRODUCTION

Alkylphenols and phthalates are xenobiotics which are abundantly distributed in the environment and are probably among the assemblage of chemicals responsible for the estrogenic effect of treated sewage effluent (Purdom *et al*., 1994; Sumpter, 1995) and certain industrial discharges (Arukwe *et al*., 1997; Knudsen *et al*., 1997). Effluents from both sources are estrogenic to fish, eliciting production of the yolk protein precursor vitellogenin (Purdom *et al*., 1994; Harries *et al*., 1997; Knudsen *et al*., 1997) and the eggshell (zona radiata) protein (Arukwe *et al*., 1997) in exposed fish. These proteins are induced by both estradiol, the endogenous piscine estrogen, and estrogenic chemicals, with the induction of zona radiata proteins occurring at lower concentrations of estrogen than are necessary to induce vitellogenin (Arukwe *et al*., 1997). Alkylphenols and phthalates are both weakly estrogenic when tested in a variety of assay systems (Pelissero *et al*., 1993; Jobling *et al*. 1995; Soto *et al*., 1995; Nimrod and Benson, 1996; Knudsen & Pottinger, 1998) and there has been some debate as to whether the relatively low levels of estrogenicity displayed by these compounds may increase when they occur in combination, as they do in the environment. However, in a recent study Knudsen and Pottinger (1998) showed that the degree to which [^{3}H]estradiol was displaced from the trout hepatic estradiol receptor (ER) by competing alkylphenols and phthalates did not increase when the competitors were presented in combination compared to the degree of displacement observed when the compounds were tested singly. These observations were interpreted as indicating that no synergism occurs between these chemicals in terms of binding to the ER. Similar observations have been reported by others (Ashby *et al*., 1997; Ramamoorthy *et al*., 1997). However, an *in vitro* receptor binding assay does not adequately reflect the situation *in vivo* because no account is taken of possible metabolic transformation of the chemicals prior to presentation to the receptor (Odum *et al*., 1997). Furthermore, a receptor binding assay cannot determine whether a competing ligand is agonistic or antagonistic to the native ligand.

The aims of the present study were twofold. First, to establish whether a representative alkylphenol (octylphenol, OP) and phthalate (butylbenzylphthalate, BBP) showed evidence of synergistic activity *in vivo*. OP and BBP are among the most abundant of the alkylphenols and the phthalates found in the environment (see TemaNord, 1997).

The second aim of the study was to investigate whether the activity of the native trout estrogen, estradiol (E), is potentiated in the presence of a xenoestrogen (OP). Most attention to date has been focused on the possible "feminizing" impact of environmental estrogens on male fish in which estradiol receptors are present (Pottinger, 1986) although blood estradiol levels remain low throughout the life cycle (Whitehead *et al*., 1978). However, with few exceptions (e.g. MacLatchy and Van der Kraak, 1995), little attention has been directed to the possibility that in female fish during the period of sexual maturation, xenoestogens acting together with endogenous estradiol could potentially lead to disturbances of the normal reproductive functions.

These aims were addressed by quantifying modulation of the hepatic ER and induction of plasma Zrp proteins in rainbow trout receiving intraperitoneal (IP) injections of OP together with BBP, or OP together with E.

MATERIALS AND METHODS

Chemicals

Octylphenol was purchased from Aldrich, 17β-estradiol from Sigma and butylbenzylphthalate from TCL.

Fish

Eighteen-month old mixed-sex juvenile rainbow trout (body weight range 35-105 g, IFE Stirling strain) were held in circular 1500 l outdoor fiberglass tanks supplied with a constant flow of lake water (25 l/min), at ambient temperature (8-10°C). The fish were fed daily with commercial pellets (1% b.w./day).

Experimental procedure

One week prior to the start of the experiment 112 rainbow trout were transferred from the holding tanks to fourteen indoor glass aquaria (100 x 50 x 70 cm), eight fish per tank. Each aquarium received a constant flow of lake water (200 ml/min) from a central reservoir in which the water temperature was maintained at 10°C using a thermostatically controlled, partially recirculating, pump system. Each tank received continuous aeration. The fish were not fed during the experiments. The test chemicals were dissolved in peanut oil (Sigma) such

that total injection volume was 300-500 μl (depending on fish weight). In group (i) fish received IP injections of estradiol (1 and 10 mg/kg b.w.) and octylphenol (5 and 50 mg/kg b.w.), singly and in combination. In group (ii) fish were injected with octylphenol and butylbenzylphthalate (both at 5 and 50 mg/kg b.w.), singly and in combination. Doses were halved in the fish receiving the combined treatments. Fish in the control tanks received IP injections of peanut oil only. Duplicate tanks containing eight fish each were used for each treatment. The fish were kept undisturbed for one week following injection and then transferred to a lethal dose of anaesthetic (2-phenoxyethanol; Sigma; 1:500). Blood samples were collected from the caudal vein of each fish into a heparinized syringe and the livers were immediately removed and frozen in liquid nitrogen. Blood samples were centrifuged and plasma was collected. Both plasma and livers were stored at -70° C until analysis. The effect on the estradiol receptor of freezing livers directly in liquid nitrogen without cryo-protection is previously evaluated and found to be negligible (Pottinger, unpublished). Fish weight, length, sex and liver weight were noted at the time of sampling.

The number of estradiol-binding sites in liver cytosols was determined from a single- point assay as described in Pottinger et al. (1994). In brief, six tubes containing 200 μl aliquots of liver cytosol were incubated for 2 h at 4°C with 100 μl of buffer containing 5 nM [^{3}H]estradiol. Non-specific binding was estimated by adding a 100-fold excess of unlabelled estradiol to three of the assay tubes. Unbound steroid was removed by the addition of 200 μl of a suspension of dextran-coated charcoal. After incubation on ice for 10 min the dextran-coated charcoal was spun down and a 300 μl aliquot of the supernatant was transferred to a scintillation vial and counted under standard ^{3}H conditions. Specific binding was calculated as the difference between binding in the total and non-specific tubes and was corrected for protein concentration. Protein concentration in the various preparations was determined according to the method of Ohnishi and Barr (1978).

Zona radiata proteins were analysed using ELISA according to Arukwe et al. (1997).

Statistical analysis

One-way analysis of variance was used to determine the effect of different treatments on both ER modulation and Zrp levels.

RESULTS

No significant tank-to-tank variation in either ER modulation or Zrp induction was found within the duplicate tanks.

(i) OP and BBP; singly and in combination

Administration of OP alone caused a significant ($P<0.01$) upregulation (~2-fold increase) of the ER in treated fish relative to the controls at both concentrations tested. No modulation of the ER was seen in the fish treated with either concentration of BBP (Fig. 1a). Similar effects were observed for Zrp induction, OP eliciting production of Zrp (~3-5 fold increase) while BBP did not. In fact, a significant decrease ($P<0.05$) in Zrp was found in fish receiving BBP (Fig. 1b). In combination, the effect of OP and BBP was additive and not synergistic for both ER modulation and induction of Zrp (Figs. 1a and b). Although there was an apparent dose-related response of Zrp in OP-treated fish in this experiment (Fig. 1b) such a relationship was not evident in any of the other groups receiving OP alone or in combination with BBP (Figs. 1b and 2b). We attribute this result to indeterminate factors associated with the experimental procedure, in particular that doses of OP were not administered in direct proportion to individual fish weights but based on the mean body weight of the experimental group as a whole.

(ii) OP and E; singly and in combination

Administration of OP and E both singly and in combination resulted in a significant (~2-fold) increase in estradiol-binding capacity relative to the control fish ($P<0.05$ - 0.01). However, no synergistic effect was seen between OP and E on ER modulation (Fig. 2a.). A significant (~6-7 fold) induction of Zrp ($P<0.01$) was also observed in response to the administration of both chemicals, but no evidence of synergy was apparent (Fig 2b.).

None of the fish employed in these studies was sexually mature. There was no significant difference in the response of male or female fish to the treatments and the data for both sexes have therefore been combined.

DISCUSSION

Our results show that no synergistic interaction occurs between OP and BBP or OP and E in terms of their effects on hepatic ER abundance and Zrp induction *in vivo.* Caution should be employed in making generalisations from these data. Taken together with the findings of Knudsen and Pottinger (1998), in which a wide range of alkylphenols and phthalates were screened for synergy at the level of receptor binding in the rainbow trout ER assay, it appears that synergy between these chemicals is unlikely to occur in trout. It must be noted that in the present study the chemicals were administered by IP-injection so metabolic transformation which may occur during gill uptake from the water into the body (Barron *et al*., 1989) is excluded. Therefore, it may be prudent to carry out experiments in which fish are exposed to the test chemicals dissolved in water before reaching a final conclusion.

Treatment with BBP did not evoke any changes in the abundance of hepatic ER, or cause any induction of zona radiata proteins, suggesting that this phthalate is not functionally estrogenic to fish. This is consistent with our observations on the potency of BBP in displacing estradiol from the trout ER *in vitro* (Knudsen & Pottinger, 1998). These observations contrast with those of Jobling *et al*. (1995) who showed that BBP was an effective competitor in the trout ER assay, promoted growth in an estradiol sensitive cell line and induced transcriptional activity in a human ER system. In fact, decreases in Zrp levels, relative to control fish, were found in fish injected with BBP. The physiological significance of this observation is not immediately apparent but may indicate an inhibitory or antagonistic effect of BBP at the level of the receptor. It has been reported that the rainbow trout ER varies from the human ER in terms of ligand specificity and sensitivity (Le Drean *et al*., 1995) which may account for differences in reported BBP activity between studies in which different assay systems were employed but does not explain the disparity for reported activity of BBP in rainbow trout ER systems.

At high concentrations BBP does bind to the trout ER (Jobling *et al*., 1995; Knudsen and Pottinger, 1998) so it remains a possibility that at higher concentrations than those used in this study, BBP could exert an effect on ER activation. However, given the water solubility (2.7 mg/l; Staples *et al*., 1997) and whole fish bioconcentration factor (9.4 ; Carr *et al*., 1997) of BBP there seems little likelihood of sufficiently high concentrations occurring in the environment to result in concentrations *in vivo* which exceed the dose administered in the

current study (50 mg/kg body weight). In sewage effluent, phthalates have been detected at concentrations no higher than 54.4 μg/l (see TemaNord, 1996), and due to dilution the actual concentration in the environment will be much lower. In a field study from Italy the highest environmental water concentration of BBP reported was 6.6 μg/l (Vitali *et al.*, 1997). It is reasonable to suggest that BBP will not pose a risk as an endocrine disruptor to fish under environmentally realistic conditions. This situation should also apply to other phthalates since BBP is reported to bind to the ER with a similar affinity (Knudsen and Pottinger, 1998), or higher affinity (Jobling *et al.*, 1995) than these related compounds.

However, as suggested by others (Jobling and Sumpter, 1993), these data indicate that alkylphenols may represent a risk as environmental estrogens to fish, and potentially to other aquatic biota. Firstly, and in contrast to BBP, OP induced both an upregulation of the hepatic ER and an increase in plasma Zrp levels. Others have also reported that alkylphenols are more estrogenic than the phthalates (Jobling *et al.*, 1995; Waller *et al.*, 1996; Knudsen & Pottinger, 1998). Secondly, they are estrogenic to fish at environmentally realistic concentrations *in vivo*. Exposure of fish to several different alkylphenols at 30 μg/l has been shown to induce vitellogenin production and cause reduced testicular growth in rainbow trout (Jobling *et al.*, 1996) and exposure of medaka (*Oryzias latipes*) to nonylphenol at 50 μg/l induced an intersex condition in male fish (Gray and Metcalfe, 1997). Levels of alkylphenolic compounds as high as 180 μg/l have been detected in rivers in the UK (Blackburn and Waldock, 1995), 60 μg/l in rivers in Switzerland (Ahel *et al.*, 1994) and 17 μg/l in a river estuary in Croatia (Kvestak *et al.*, 1994). In particular, since high concentrations of alkylphenolic compounds are found in estuaries (Kvestak *et al.*, 1994) it is reasonable to support the assertion that these compounds could impose a risk as environmental estrogens, a risk which may not be restricted to areas close to outlets of municipal and industrial effluence.

Administration of estradiol evoked both an upregulation of the ER and an induction of Zrp production, a result which is consistent with other studies (MacKay *et* al., 1996; Donohoe & Curtis; 1996; Arukwe *et al.*, 1997). The induction of rainbow trout liver ER *in vitro* (Flouriot *et al.*, 1995) and *in vivo* (Donohoe & Curtis, 1996) is an estrogen-dependent phenomenon and has previously been employed to demonstrate the estrogenicity of xenobiotics. In the present study no synergistic enhancement of ER upregulation was observed when E and OP were

administered to trout in combination, relative to the effects of the compounds alone, suggesting that no potentiation of E-stimulated processes would occur in female fish in the presence of OP. However, since OP is estrogenic *in vivo*, effects on female (and male) fish may still occur, possibly manifested as inappropriate activation of E-dependent functions. It is interesting to speculate that effects of exposure to environmental estrogens may be exacerbated by the ability of the compounds to up-regulate the ER thereby enhancing their impact. This represents another aspect of the mode of action of environmental estrogens which is not amenable to investigation in many *in vitro* assay systems.

To our knowledge, the present study is the first report of alterations in ER abundance *in vivo* in response to OP treatment. However, OP is known to bind to both the human and trout ER (White *et al*., 1994; Knudsen and Pottinger, 1998), and to cause production of vitellogenin both *in vivo* (Jobling *et al*., 1996) and *in vitro* (Jobling and Sumpter, 1993; White *et al*., 1994). OP-induced upregulation of the ER is therefore unsurprising. Other xenobiotics, for example DDT metabolites, are known to cause an upregulation of the hepatic ER in rainbow trout *in vivo* (Donohoe and Curtis, 1996) and nonylphenol induces rainbow trout ER mRNA *in vitro* (Flouriot *et al*., 1995) and *in vivo* (Arukwe *et al*., in prep.) suggesting that ER upregulation is an effective indicator of the estrogenic effect of xenobiotics. However, the changes in ER abundance were less pronounced than the degree of Zrp induction observed in fish in the present study, suggesting that Zrp induction may be a more sensitive indicator of estrogenicity than ER modulation. This is in agreement with the findings of Donohoe and Curtis (1996) who demonstrated that the vitellogenin response was more substantive than the upregulation of the ER in fish treated with DDT metabolites.

Both alkylphenols and phthalates have been suggested as potential causes of the estrogenic effects of treated sewage effluent on fish in the UK (Sumpter, 1995) and could also be responsible for the estrogenic effect of treated sewage and effluents from oil-industry in Norway (Arukwe *et al*., 1997; Knudsen *et al*., 1997). Given the low estrogenicity of alkylphenols and phthalates and the evident lack of synergy between them, it is reasonable to question the role of these chemicals as the *main* cause of the estrogenic effect of such effluents. Recently, the estrogenicity of sewage effluents in UK waters has been attributed to the presence of natural estrogens, probably of human origin (Anon, 1997), and this is perhaps

a more likely explanation for the estrogenic effects of non-industrial effluents on fish than the presence of xenobiotic compounds.

In conclusion, the results of the present study suggest that no synergistic effects are observed *in vivo* in fish exposed to octylphenol and butylbenzylphthalate in combination, or octylphenol and estradiol in combination. Furthermore, it is argued that phthalates do not constitute a risk to fish as environmental estrogens, while further data are presented which confirm that alkylphenols do exert estrogenic effects on fish and these effects extend to modulation of the ER and inappropriate production of yolk envelope proteins.

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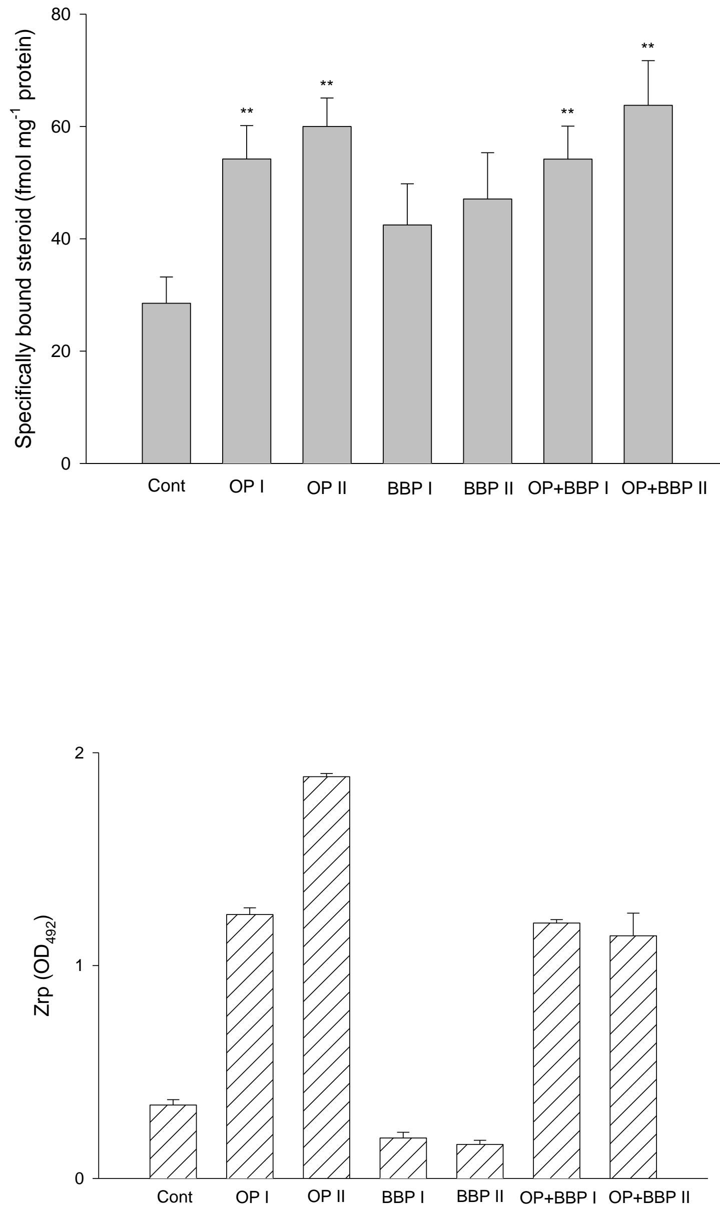

Fig. 1. (a) Changes in the abundance of hepatic estradiol receptor in rainbow trout given injections of octylphenol (OP) and butylbenzylphthalate (BBP), or OP + BBP, at two different concentrations (I: 5 mg/kg body weight; II: 50 mg/kg body weight) alone or in combination. Doses were halved in the fish receiving the combined treatments. (b) Induction of Zrp in the same fish expressed as the mean ELISA absorbance values. Each bar represents the mean of 16 fish $\pm$ SEM. Significant differences from the control value are denoted by *P<0.05 and ** P<0.01.

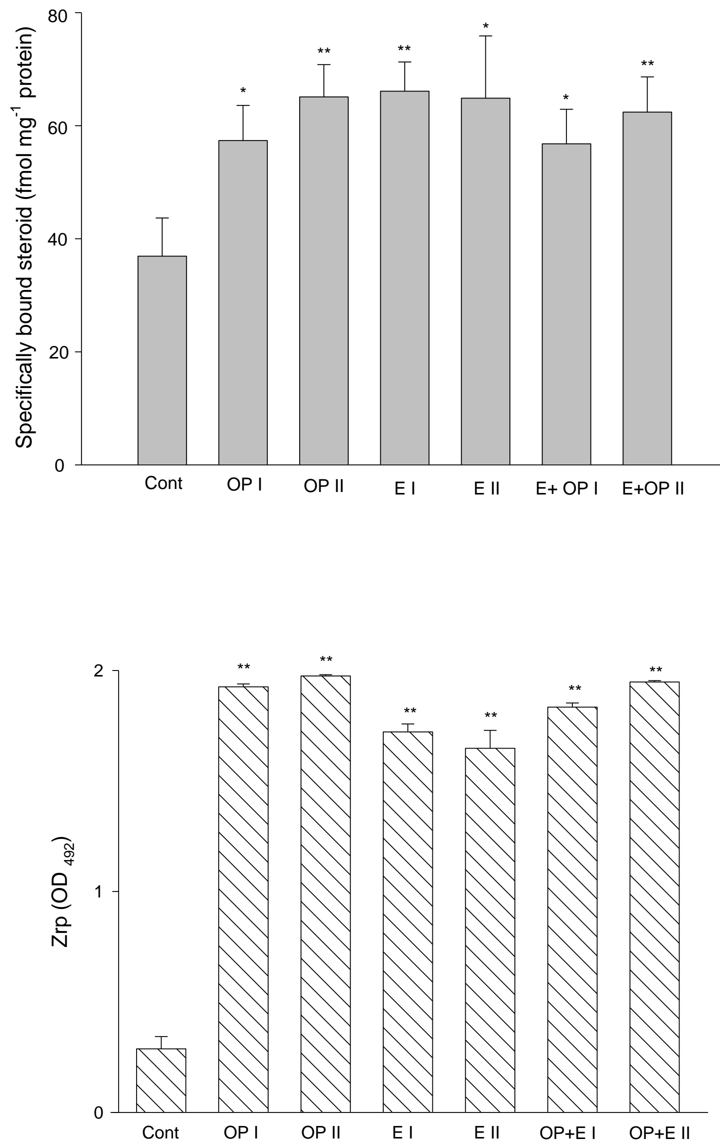

Fig 2. (a) Changes in the abundance of hepatic estradiol receptor in rainbow trout given injections of octylphenol (OP) and estradiol (E), or OP + E, at two different concentrations (OPI: 5 mg/kg body weight; OPII: 50 mg/kg body weight; EI: 1 mg/kg body weight; EII: 10 mg/kg body weight) alone or in combination. Doses were halved in the fish receiving the combined treatments.(b) Induction of Zrp in the same fish expressed as the mean ELISA absorbance values. Each bar represents the mean of 16 fish $\pm$ SEM. Significant differences from the control value are denoted by * $P<0.05$; ** $P<0.01$.