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Joint anti-estrogenic effects of PCP and TCDD in primary cultures of juvenile goldfish hepatocytes using vitellogenin as a biomarker

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Abstract

This work evaluated the joint anti-estrogenic effects of pentachlorophenol (PCP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) against 17β-estradiol (E2) in juvenile goldfish (*Carassius auratus*) hepatocyte cultures. The level of vitellogenin (VTG) as a biomarker was determined by exposing hepatocytes to individual E2, PCP and TCDD, as well as to E2 in the presence of PCP, TCDD or their mixtures of various concentrations. PCP and TCDD did not exhibit estrogenicity. Both chemicals reduced the estrogenicity of E2, indicating the anti-estrogenic effects of PCP and TCDD. Their anti-estrogenic EC_{50} values were calculated. The joint anti-estrogenic effects against E2 increased with increasing the PCP-to-TCDD ratio of mixture. Marking's indices were <0, suggesting an antagonism in anti-estrogenic effects between PCP and TCDD. The anti-estrogenic effects of PCP appeared to result primarily from the competitive binding to estrogen receptor. While TCDD may undergo an indirect binding process for its anti-estrogenic effects, the accurate mechanisms remain to be understood. The observed antagonism in anti-estrogenic effects resulted apparently from the mutual inhibition by PCP and TCDD.

Keywords: PCP; TCDD; VTG; Joint estrogenic effects

1. Introduction

While the environmental effects of individual pollutants on organisms in ecosystems have been well-documented, joint effects of multi-pollutants cannot be simply predicted by single-factor data in most cases and hence remain poorly understood. Simultaneous or sequential exposure of organisms to a variety of pollutants may result in an additive, synergistic, or antagonistic effect of the observed separate exposures. The joint effects can be determined by the following equation:

$$S = m_A \cdot EC_{50}/n_A \cdot EC_{50} + m_B \cdot EC_{50}/n_B \cdot EC_{50}$$

where EC_{50} is the effective concentration required to bring about a 50% change, m_A and m_B are the concentrations of pollutants A and B in the mixture, respectively, and n_A and n_B are the concentrations of single A and B, respectively (John and Line, 2002). Although the research on the joint effects of pollutant mixtures has been constantly rising during the past several decades, information on the joint estrogenic effects of chemicals remains scanty (Liliane et al., 2002).

It is known that chemicals that mimic 17β-estradiol (E2), an endogenic and the most potent estrogen, are able to induce the synthesis of vitellogenin (VTG), a yolk phospholipoglycoprotein, in the livers of multiple oviparous species such as fish. VTG is produced in response to E2 stimulation in female oviparous animals. Under normal

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conditions with very low level of E2 in male and juvenile fish, no significant level of VTG can be detected. Nevertheless, the VTG gene is present in male and juvenile fish, and can be triggered by administration of E2 (Purdom et al., 1994; Kundsen et al., 1997). High levels of VTG in male and juvenile fish are therefore indicative of exposure to estrogens or chemicals that are able to act like estrogens (Sumpter, 1995). As male and juvenile fish are highly sensitive to estrogens in the environment, VTG can be used as a biomarker for exposure to estrogenic chemicals. Among many existing methods for evaluation of the estrogenic effects, the test of the induction of VTG synthesis in hepatocytes not only helps to study the signaling mechanisms of xenoestrogens, but also provides a rapid screening tool for evaluating the estrogenic potential of environmental contaminants (Sumpter and Jobling, 1995).

Pentachlorophenol (PCP) has been extensively used as a biocide in fishery, agriculture and industry. In many freshwater lakes of China, a large quantity of PCP has been sprayed for control of oncomelanias to prevent a schistosomiasis epidemic. Although PCP was banned for further use beginning in 1990, its persistence resulted in a long-lasting contamination of aquatic environment (Zheng et al., 2000). Studies showed that PCP possesses endocrine-disrupting functions (Flouriot et al., 1995; Louise and Gerald, 1996; Benjamin et al., 2002) and now is identified to be an endocrine-disrupting chemical (EDC) by USEPA. In addition, PCP often contains a trace amount of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), another well-known EDC (Safe, 1995; Smeets et al., 1999; Wan et al., 2004). The joint anti-estrogenic effects of co-existing PCP and TCDD have, however, not yet been reported. The aim of this study was therefore to assess the joint anti-estrogenic effects of PCP and TCDD in primary cultures of juvenile goldfish hepatocytes using VTG as a biomarker.

2. Material and methods

2.1. Chemicals and goldfish

E2 (98% pure) and tamoxifen (98%) were purchased from Sigma; PCP (99%) and TCDD (99%) were obtained from Fisher. E2, tamoxifen and PCP were prepared in ethanol as 1 mg/ml stock solutions and stored at −20 °C. TCDD was dissolved in dimethylsulfoxide at the concentration of 1 mg/ml and stored at 4 °C. Prior to use, all stock solutions were diluted to desired concentrations in phenol red-free experimental medium. The final solvent concentration in the medium did not exceed 0.1% (v/v); this concentration did not affect the hepatocyte VTG induction. The polyclonal antibody raised against carp VTG was kindly provided by Professor Ying Xu of the Institute of Hydrobiology, Chinese Academy of Sciences. Fish used in the experiments were genetically uniform juvenile goldfish (*Carassius auratus*) (1.6 ± 0.4 g each) purchased from Beiyuan Market in Beijing. All healthy fish were domesticated at 20 ± 1.0 °C in an aquatic trunk filled with dechlorinated tap water for 7 days and fed once per day.

2.2. Isolation of hepatocytes

Hepatocytes were isolated according to the method described previously (Sonia et al., 2004). Fish skin was sterilized by alcohol and its abdomen was dissected with sterile instruments from anus toward head. Liver tissue was excised and rinsed twice within 5 min with a buffer solution free of Ca^{2+} and Mg^{2+} (pH 7.5) but containing 0.145 mol/l NaCl, 5.4 mmol/l KCl, 5 mmol/l EDTA, 1.1 mmol/l KH_2PO_4, 12 mmol/l $NaHCO_3$, 3 mmol/l NaH_2PO_4, and 100 mmol/l HEPES. The liver tissue was then minced into pieces and transferred to a 50-ml conical tube, to which a solution of 0.25% trypsin was added. The tube was shaken on a shaker at 200 rpm for 5 min to obtain the cell suspension, which was then filtered through a 200-mesh sieve. The cell suspensions were pooled and centrifuged at 1000 rpm for 2 min; the cell pellet was washed with phenol red-free 1640 (Invitrogen) (pH 7.4) containing 10% steroid-free foetal bovine serum. The cells were resuspended in the culture medium and counted. Their viability was >90% as assessed with typan blue exclusion. Hepatocytes were seeded in 24-well tissue culture plates at 1×10^6 cells/ml. The culture was supplemented with 1 μmol/l insulin, 10 μmol/l hydrocortisone, and 10% steroid-free foetal bovine serum. One day after the isolation, hepatocytes were attached to the wells and formed a monolayer of 50–60% confluency. At this time, the medium was removed and replenished with fresh medium containing test chemical(s) or solvent only. Hepatocytes were exposed for 120 h and the medium was changed every 36 h. In order not to dislodge the cells during the medium replacement, only 90% of the used medium was removed. The medium was transferred into 96-well plates, frozen, and kept at −70 °C until analysis for VTG content. Each concentration of a chemical was tested in 6 wells in one plate.

2.3. VTG determination by enzyme-linked immunosorbent assay (ELISA)

VTG production of hepatocytes was quantified by means of ELISA according to Zhong et al. (2004) with a few minor modifications, in which a polyclonal rabbit antibody was used against carp (*Cyprinus carpio*) VTG. This antibody has been shown to bind to VTG produced by other cyprinid species such as fathead minnow and goldfish (Tyler et al., 1996). Medium samples were diluted in a phosphate buffer solution (PBS, 0.14 mol/l NaCl, 0.015 mol/l KH_2PO_4, 0.075 mol/l Na_2HPO_4, 0.027 mol/l KCl; pH 7.3). Diluted samples were pipetted in triplicate into 96-well culture plates (100 μl/well). The plates were covered and incubated overnight at 4 °C. The wells were then washed three times with 200 μl of PBS, followed by incubation in 200 μl of PBS-3% BSA for 5 h at 4 °C (blocking step). Next, the plates were again washed three times with 200 μl of PBS,