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AhR-mediated and antiestrogenic activity of humic substances

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Abstract

Humic substances (HS) were for decades regarded as inert in the ecosystems with respect to their possible toxicity. However, HS have been recently shown to elicit various adverse effects generally attributed to xenobiotics. In our study, we used MVLN and H4IIE-*luc* cell lines stably transfected with luciferase gene under control of estrogen receptor (ER) and Ah receptor (AhR; receptor connected with so-called dioxin-like toxicity) for assessment of anti/estrogenic and AhR-mediated effects of 12 commercially available humic substances. Out of those, five humic acids were shown to induce AhR-mediated activity with relative potencies related to TCDD 2.6×10^{-8}–7.4×10^{-8}. Organic extracts of HS solutions also elicited high activities what means that lipophilic molecules are responsible for a great part of effect. However, relatively high activity remaining in extracted solution suggests also presence of polar AhR-agonists. Contribution of persistent organic compounds to the observed effects was ruled out by H_2SO_4 treatment. Eight out of twelve HS elicited significant antiestrogenic effects with IC_{50} ranging from 40 to 164 mg l^{-1}. The possible explanations of the antiestrogenic effect include sorption of 17-β-estradiol (E2) on HS, changes in membrane permeability for E2 or another specific mechanism.

Keywords: Ah receptor; AhR-mediated activity; Antiestrogenic effects; Endocrine disruption

1. Introduction

Humic substances (HS) are ubiquitous natural products of decomposition of dead organic matter. Generally, HS can be divided into three groups: humic acids (HA), fulvic acids (FA) and humins. In the aquatic environment, HS form approximately 50–70% of dissolved/natural organic matter (D/NOM, Timofeyev et al., 2004), which is in most natural freshwaters in the concentration range 0.5–50 mg l^{-1} (Steinberg, 2003).

Until recently, only indirect effects of HS in the ecosystems such as changes in bioavailability of organic pollutants and/or complexation of inorganic ions, particularly heavy metals, have been reported (Steinberg, 2003). The presumed large molecules of HS were not considered to be able to penetrate into cells. However, recent studies focused on HS uptake showed that at least parts of natural as well as artificial HS can be assimilated by plant cells in culture (Wang et al., 1999) as well as crustaceans (*Gammarus pulex*) or even vertebrates (*Rana arvalis* tadpoles; Steinberg et al., 2003). Furthermore, biological effects of HS have been reported for algae, invertebrates as well as vertebrates (Pflugmacher et al., 2001; Steinberg et al., 2003).

Direct toxic effects of HS were reported for the freshwater snail *Lymnea stagnalis*. Exposure to natural concentrations of HS from the Suwannee River (0.5 mg l^{-1}) caused death of up to 20% of individuals (Steinberg et al., 2003). Responses of nonspecific markers of toxic effects of HS were also observed. Elevated levels of detoxification enzymes glutathione-*S*-transferase and glutathione

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peroxidase were reported in crustaceans *Daphnia magna* following HS treatment at concentration range 0.5–100 mg l^{-1} (Wiegand et al., 2003). An increase in peroxidase activity as well as release of heat shock proteins was also observed in several amphipod species (Timofeyev et al., 2004) and carp (Wiegand et al., 2003). Hoss et al. (2001) reported that natural organic matter (NOM) altered reproduction of nematodes *Caenorhabditis elegans* what was further confirmed in a study with broader spectra of HS (Steinberg et al., 2002).

A synthetic humic substance HS1500 (a polyphenol oxidation product) was shown to elicit significant estrogenic and anti-thyroidal effects on *Xenopus laevis* tadpoles (Lutz et al., 2005). Furthermore, long-term exposure of swordtail fish *Xiphophorus helleri* fry to a high concentration of HS1500 (from 5 to 180 mg l^{-1}) lead to changes of sex ratio in favor of females as well as to body mass increase in both males and females (Meinelt et al., 2004), while survival of fry has not decreased even at exposure to 500 mg l^{-1} (Steinberg et al., 2003). However, the same very high concentration of HS1500 has significantly decreased the survival rate of the exposed embryos of fish *Danio rerio*, whereas exposure to lower concentrations of 5–50 mg l^{-1} slightly increased the survival rate of embryos relative to the control (Steinberg et al., 2003).

In spite of these *in vivo* experiments, no mechanistic *in vitro* studies had been conducted to determine the mode of action of direct effects. With respect to observed estrogenic effects as well as increase in levels of detoxification enzymes, we decided to investigate possible interactions of a wide spectrum of HS (including HA, FA and NOM obtained from various sources) with estrogen receptor (ER) and aryl hydrocarbon receptor (AhR). The latter is responsible for so-called dioxin-like mode of action resulting in a wide range of *in vivo* effects in vertebrates including, e.g. enormous enhancement of activity of detoxification enzymes.

2. Material and methods

2.1. Chemicals

HS isolated from different matrices were purchased from various sources: HA-Fluka (Product No. 53680; Fluka, Switzerland), HA sodium salt (Product No. H16752; Sigma–Aldrich, Prague, Czech Republic). The following reference HS were purchased from the International Humic Substances Society (IHSS, Denver, USA): Suwannee River HA (Product No. 2S101H), Suwannee River FA (1S101F) and Suwannee River NOM (1R101N), Florida Peat HA (1S103H) and Florida Peat FA (2S103F), Nordic Aquatic FA (1R105F), Nordic Reservoir NOM (1R108N), Waskish Peat HA (1R107H), Elliot Soil HA (1S102H) and Leonardite HA (1S104H). Reference compounds were purchased from Dr. Ehrenstorfer, Augsburg, Germany (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) and Sigma–Aldrich (17-β-estradiol, E2).

2.2. Sample preparation

HS were dissolved in 0.05 M NaOH and final concentrations ranging from 1.9 to 150 mg l^{-1} were studied. To evaluate the nature of active molecules, further investigations were conducted. The alkaline solution of HS was extracted three times with a 3:1 hexane:dichloromethane mixture. Half of this extract was transferred into DMSO for *in vitro* assays (further regarded as organic extract); the other half was analyzed using GC–MS for known xenobiotics such as seven indicator PCB (IUPAC No. 28, 52, 101, 118, 138, 153 and 180) and 16 PAH monitored by the United States Environmental Protection Agency (USEPA). To determine possible contributions of other persistent organic compounds that might have been present in standard materials, the alkaline solutions (200 μl) were transferred into excess of 96% H_2SO_4 (5 ml) and liquid–liquid extraction with hexane:dichloromethane (3×5 ml) was conducted. Using this method, only persistent compounds such as PCDD, PCDF and PCB were extracted. The original alkaline solutions were examined for both AhR-mediated and anti/estrogenic activity, organic extracts as well as extraction residues of alkali solutions and H_2SO_4-treated extracts were tested for AhR-mediated activity.

2.3. In vitro assays

H4IIE-*luc* (rat hepatocarcinoma) and MVLN (human breast carcinoma) cells stably transfected with luciferase gene under control of AhR and ER, respectively, were used for analysis of receptor activation. Both cell lines are well established models for evaluation of AhR-mediated (H4IIE-*luc*) and anti/estrogenic (MVLN) activities of pure substances as well as environmental samples (Demirpence et al., 1993; Machala et al., 2001; Villeneuve et al., 2002).

H4IIE-*luc* cells: Cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (both PAA laboratories, Pasching, Austria) at 5% CO_2 and 37 °C. Once the cells reached about 70% confluence they were passaged and seeded into a sterile 96-well plate at density 15000 cells/well. After 24 h, the cells were exposed in triplicates to the tested samples (concentrations 1.9, 5.6, 16.7, 50 and 150 mg l^{-1}) or reference compound (dilution series 0.1–100 pM TCDD) for 24 h at 37 °C (final vehicle concentration was 0.5% v/v). Cells exposed to DMEM with 0.5% DMSO and 0.5% 0.05 M NaOH were used for the appropriate vehicle controls. Intensity of luciferase luminescence was measured using Promega Steady Glo Kit (Promega, Mannheim, Germany) after 24 h exposure. At least three independent assays have been conducted for each concentration tested.

MVLN cells: Cells were grown in DMEM-F12 without phenol red (Sigma Aldrich, USA) containing 10% fetal calf serum. For experiments, cells were grown in medium containing fetal calf serum treated with dextran-coated charcoal (strongly reduced concentrations of natural steroids). For studies of anti/estrogenic effects of HS, cells were