Activation of Ah Receptor by Pure Humic Acids

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ABSTRACT: Humic substances (HS) are ubiquitous in the environment. However, some studies indicate that HS could induce direct adverse effects on human health and hormone-like effects in fish, amphibians, and invertebrates. In this study we investigated a possible biochemical mechanism of HS toxicity via activation of the aryl hydrocarbon receptor (AhR). AhR mediates the toxic and biological effects of environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), but a number of structurally diverse compounds has also been found to activate AhR. Alkali solutions of humic acids (HA) were prepared, and subsequently, lipophilic compounds (including parts of HA) were extracted by liquid–liquid extraction with hexane/dichloromethane. Organic extract of HA was further treated with sulfuric acid to study the role of possible trace persistent contaminants. In vitro dioxin-like activities of obtained extract and HA solutions have been evaluated using H4IIE.luc cells by determining the ethoxyresorufin-O-deethylase (EROD) activity and induction of AhR-dependent reporter luciferase. Traces of nonpersistent residues in HA with known AhR activity were identified and quantified by GC-MS. Our results show that an alkali solution as well as organic extract of HA were active in both EROD and luciferase assays, while H2SO4-treated extract activity was negligible. Only nonsignificant levels of AhR-inducing contaminants (PAHs and PCBs) were found in the HA samples. Our results indicate that HA or their fragments can elicit significant inductions of AhR-mediated effects in vitro. To our best knowledge, this study is the first in providing direct evidence of dioxin-like effects of HA. Further efforts should focus on detailed characterization of potential toxic effects of various HSs.

Keywords: aryl hydrocarbon receptor; H4IIE.luc; luciferase; EROD; humic substances

INTRODUCTION

Humic acids (HA) and fulvic acids are certain fractions of the group of organic compounds called humic substances (HS). HS are ubiquitous organic compounds that occur in soils and aquatic ecosystems. In aquatic ecosystems, they are the main components of dissolved organic matter that is found in most natural freshwaters in concentrations 0.5–50 mg L⁻¹ (Steinberg, 2003). Until recently, HS were generally considered to be inert and only the sorption of metals and organic contaminants on HS was considered to be important (McCarthy, 1987; McGeer et al., 2002; Mezin and Hale, 2004). However, some recent works have shown that HS can act as xenobiotic chemicals. Meinelt et al. (2001) found that synthetic HA (HS1500) affects physiological condition and slightly also the sex ratio of the fish Xiphophorus helleri. Furthermore, the hormone-like effect of HS on nematode Caenorhabditis elegans (Hoss et al., 2001;
Steinberg et al., 2002), and increase in amphipod mortality and change of biochemical parameters (Timofeyev et al., 2004) were shown. Recent studies conducted in Taiwan have suggested that HS can even be the causative agents of endemic human diseases such as Blackfoot disease (Cheng et al., 1999; Gau et al., 2001; Hseu et al., 2002; Huang et al., 2003) or Kashin-Beck disease (Liang et al., 1999).

So far, two types of mechanisms of HS action have been studied. Nonspecific mechanisms of action were examined with Daphnia magna, three other amphipod species, and common carp Cyprinus carpio (Wiegand et al., 2003). Dose–response relationships between HS and/or natural organic matter (NOM) exposure and modulation of heat shock proteins 70 (Hsp 70) were found in carp. Additionally, HS induced Hsp 70 as well as the biotransformation enzymes glutathione S-transferases, oxidative-stress enzymes peroxidase and glutathione-peroxidase in amphipods and D. magna (Wiegand et al., 2003). A more specific mechanism of action is modulation of photosynthetic oxygen release induced by HS and NOM in alga Scenedesmus armatus, water moss Vesicularia dubyana, and hornwort Ceratophyllum demersum (Pflugmacher et al., 1999; Steinberg et al., 2003).

Another specific mechanism of direct action of HS, the activation of cytosolic aryl hydrocarbon receptor (AhR), was suggested by Pflugmacher (unpublished results, from Steinberg, 2003). The hypothesis of binding interaction between HS and AhR was derived from indirect evidence that Hsp 70 might be induced after activation of the AhR. However, we are not aware of any research evidence of this fact. The aim of this study was to examine whether the HA is able to act as an activator of the AhR. For this purpose, we used two different endpoints (reporter luciferase and ethoxyresorufin-O-deethylase (EROD) assays) for determination of the ability of HA to elicit AhR-mediated response in vitro in H4IIE.luc cell line. The advantage of the EROD assay is a natural AhR-mediated expression of measured protein activity, while the expression of luciferase, being foreign to the cell, is probably not affected by posttranscriptional and -translational events which influence CYP1A1 expression measured as EROD (Sanderson et al., 1996).

### MATERIALS AND METHODS

#### Materials

Humic acids (HA) was purchased from Fluka, Switzerland (prod. no. 53680). HA dissolved in 0.05 M NaOH (“alkali HA”) was used for testing. Extraction of lipophilic organic compounds from alkali HA solution (fragments of HA, but also possible AhR-active organic contaminants like PCDDs, PCDFs, PCBs, PAHs, etc.) was carried out by liquid–liquid extraction (LLE; 3 mL of alkali HA solution, 10 g L⁻¹), was extracted with 5 mL of hexane/dichloromethane, 3:1 v/v). This LLE was repeated three times and all organic extracts were pooled and concentrated under a stream of nitrogen to a volume of 1 mL (“HA-extract”). One half of the concentrated organic extract was transferred into 1 mL dimethyl sulfoxide (DMSO) and prepared for bioassay analysis; another half was analyzed for the presence of 16 US EPA priority PAHs using GC-MS (Hewlett-Packard, USA) and 7 indicator PCBs (IUPAC No. 28, 52, 101, 118, 138, 153, and 180) using GC-ECD (Hewlett-Packard, USA). One-milliliter aliquot of remaining alkali HA solution after extraction (“alkali HA remaining after LLE”) was also stored for subsequent bioassay analysis. An extraction of only persistent organic compounds (e.g., PCDDs, PCBs, and PCDFs) from alkali HA was conducted in a manner similar to that described earlier, but with the addition of 5 mL of H₂SO₄ to the extraction mixture with only 0.25 mL of HA (i.e., HA + H₂SO₄ + hexane/DCM) and incubation of the mixture in water bath. The scheme of preparation for all of the tested samples is shown in Figure 1. Appropriate extraction control samples were prepared to preclude any possible contamination with AhR activators from the used solvents or equipment.

#### Cell Culture and Exposure

The H4IIE.luc cells are stably transfected with DRE-driven firefly luciferase reporter gene construct; its transcriptional activation occurs in an AhR-dependent manner (Sanderson et al., 1996). Recombinant rat hepatoma H4IIE.luc cells were grown and maintained in DMEM medium containing 10% fetal calf serum (PPA laboratories, Austria) at 5% CO₂ and 37 °C. Cells were grown to about 70% confluence in a sterile 96-well plate for 24 h, and subsequently incubated with reference tetrachlorodibenzop-dioxin (TCDD) and tested samples for up to 24 h at 37 °C (final volume of vehicle was 0.5% v/v). Cells exposed to DMEM with 0.5% DMSO or 0.5% 0.05 M NaOH were used for the appropriate vehicle controls.

![Fig. 1. Preparation scheme of tested solutions as described in Materials and Methods.](image-url)
Luciferase Reporter Gene Assay and EROD Activity

After incubation with test substances, cells were washed twice with phosphate-buffered saline, and luminescence was measured with Steady-Glo Kit (Promega, USA) according to manufacturer's instructions, using an automated microplate fluoro-/luminometer GENios (TECAN, Switzerland). Final values are expressed as percentage of maximal TCDD induction (after subtraction of the solvent control activity). EROD activity was measured by a procedure slightly modified from methods described by Jung et al. (2001). We measured fluorescence at 550 nm excitation and 612 nm emission. Fluorescence was measured using a microplate fluoro-/luminometer GENios.

Statistical Analysis

Data were examined statistically using Statistica for Windows 6.0 (StatSoft, USA) and a level of significance $P = 0.05$. In figures, mean ± SD of triplicate determinations are shown. Simple log-linear regression models were calculated for linear portions of the dose–response curves of standard TCDD and tested HA samples. Relative luciferase-induction potency of HA (expressed as relative potency, REP) was calculated using the equi-effective approach (Jones and Anderson, 1999; Villeneuve et al., 2002). Concentrations of the HA inducing 50% of the TCDD-max response ($C_{EQ-50}$) were compared with the $EC_{50}$ of the reference TCDD, and the $REP_{HA} = C_{EQ-50}/EC_{50}$ was derived for the HA. For risk characterization of AhR-active compounds concept of toxic equivalents (TEQ, Van der Berg et al., 1998) was used. Bioassay-determined TEQs of HA (bioassay-TEQ$_{HA}$) were calculated using the equation bioassay-TEQ$_{HA} = [HA_{Fluka}] \times REP_{HA}$. Chemical analyses-derived TEQs (chem-TEQ) of determined PAHs were calculated using the equation $\text{chem-TEQ} = \Sigma [\text{PAH}_i \times \text{REP}_i]$, using the REP values for individual PAHs suggested by Machala et al. (2001).

RESULTS AND DISCUSSION

The three greatest tested concentrations of HA (5, 15, and 50 mg L$^{-1}$) significantly increased luciferase reporter gene activity in H4IE.luc cells (Fig. 2) in a dose-dependent manner. Activity was related to reference TCDD and resulting REP of HA determined by luciferase induction assay equaled $6 \times 10^{-8}$. HA extract elicited practically an identical luciferase response as the original alkali HA (Fig. 2). This indicates that almost all of the AhR activators present in HA were extracted into the organic phase. Induction of luciferase activity at maximal tested concentration of alkali HA remaining after LLE was about 3.5-fold less than the activity induced by the original alkali HA, but still significantly greater than control sample (Fig. 2). However, dioxin-like activity of alkali HA and HA extract were practically identical. Accordingly, we assume that removal of extracted parts of HA has broken the balance in HA solution and thus other AhR-activating compounds could be released from the complex HA. It was not possible to determine the REP of alkali HA remaining after LLE because even the greatest tested concentration (50 mg L$^{-1}$) did not achieve 50% maximal standard induction. Data obtained from EROD assay (Fig. 3) corresponded very well with the results from luciferase assay.

The structure of humic substances (HS) studied using multidimensional NMR is presented as a macromolecular aggregate composed from a mixture of relatively low-molecular weight (<2kDa) organic compounds holding together through a complex combination of hydrophobic,
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charge-transfer and hydrogen bond interactions, and metal bridging (Simpson et al., 2002). This finding, in conjunc-
tion with our results, implies that activation of AhR in H4IIE.luc cells could be caused mainly by lipophilic parts of macromolecular “humic aggregate” that were released from HA macromolecule.

Possible false-positive results caused by the contamination of HA with classical AhR activators (such as PCDDs, PCDFs, PCBs, and PAHs) were investigated by both H2SO4-treatment of HA extract as well as chemical analyses. No significant luciferase activity of HA extract after H2SO4 treatment ruled out possible contribution of persistent AhR activators to observed luciferase activity of alkali HA and HA extract. The bioassay data were confirmed by chemical analyses. None of the 7 indicator PCB congeners was detected (<LOD), and only trace concentrations of some PAHs known to activate AhR were determined (concentrations in ng g⁻¹): Fluoranthene-80, Pyrene-376, Indeno (123cd)pyren-160. Chem-TEQ calculated from chemical data for detected PAHs equaled 4.8 × 10⁻² ng TCDD g⁻¹, what accounted for only about 0.1% of total observed AhR-mediated activity of HAFluka (bioassay-TEQHA ~ 60 ng TCDD g⁻³).

The ability of alkali HA and HA extracts to induce AhR observed in our study is, at the first sight, surprising because HA (or their fragments) has apparently different molecular structure than the prototypical and most potent AhR agonists such as PCDDs, PCDFs, PCBs, or PAHs (Safe, 1990; Simpson et al., 2002). However, several studies have revealed AhR activators with very diverse structures (Denison and Heath-Pagliuso, 1998; Denison and Nagy, 2003; Jeuken et al., 2003), and consequently AhR might be considered as a relatively nonspecific receptor.

In conclusion, the results of our study have shown that both alkali HA and its organic extract cause significant inductions of AhR-mediated responses in vitro (as shown by both reporter luciferase inductions and EROD bioassays). Possible false-positives due to the presence of AhR-active contaminants were excluded by chemical analyses. In vitro dioxin-like effects of HA were observed at environmentally relevant concentrations and the determined REP₇₄₈ was 6 × 10⁻⁸. Therefore, our study indicates that HS (or their constituents) might act as AhR-inducing substances in vivo, and can thus significantly affect various toxicological processes. However, HS are not uniform compounds: their structure and properties differ with regard to the place of origin. Our further experiments focus on detailed characterization of AhR modulations by a wider range of available HS.

REFERENCES


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