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Contamination with retinoic acid receptor agonists in two rivers in the Kinki region of Japan

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ABSTRACT

This study was conducted to investigate the agonistic activity against human retinoic acid receptor (RAR) α in the Lake Biwa–Yodo River and the Ina River in the Kinki region of Japan. To accomplish this, a yeast two-hybrid assay was used to elucidate the spatial and temporal variations and potential sources of RARα agonist contamination in the river basins. RARα agonistic activity was commonly detected in the surface water samples collected along two rivers at different periods, with maximum all-*trans* retinoic acid (atRA) equivalents of 47.6 ng-atRA/L and 23.5 ng-atRA/L being observed in Lake Biwa–Yodo River and Ina River, respectively. The results indicated that RARα agonists are always present and widespread in the rivers. Comparative investigation of RARα and estrogen receptor α agonistic activities at 20 stations along each river revealed that the spatial variation pattern of RARα agonist contamination was entirely different from that of the estrogenic compound contamination. This suggests that the effluent from municipal wastewater treatment plants, a primary source of estrogenic compounds, seemed not to be the cause of RARα agonist contamination in the rivers. Fractionation using high performance liquid chromatography (HPLC) directed by the bioassay found two bioactive fractions from river water samples, suggesting the presence of at least two RARα agonists in the rivers. Although a trial conducted to identify RARα agonists in the major bioactive fraction was not completed as part of this study, comparison of retention times in HPLC analysis and quantification with liquid chromatography–mass spectrometry analysis revealed that the major causative contaminants responsible for the RARα agonistic activity were not RAs (natural RAR ligands) and 4-oxo-RAs, while 4-oxo-RAs were identified as the major RAR agonists in sewage in Beijing, China. These findings suggest that there are unknown RARα agonists with high activity in the rivers.
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1. Introduction

There has been great concern regarding environmental contaminants that disrupt or alter functions of the endocrine system primarily by binding to nuclear receptors and subsequently causing detrimental effects in intact organisms. The occurrence and fate of these so-called endocrine disrupting chemicals in the aquatic environment have been extensively studied while mainly focusing on compounds that interfere with steroid hormone receptors such as estrogen receptor (ER) and androgen receptor (Campbell et al., 2006; Sharma et al., 2009). However, it has been shown that the nuclear receptor superfamily contains many different receptors in eukaryotic organisms (Chawla et al., 2001), and each member of the nuclear receptor superfamily can mediate endocrine disruptive effects (Janošek et al., 2006; Tabb and Blumberg, 2006).

Retinoic acid (RA) receptors (RARs) are members of the nuclear receptor superfamily whose specific natural ligands are all-*trans* RA (atRA) and 9-*cis* RA (9cRA) derived from retinoid (vitamin A) precursors (Chambon, 1996). RAs are essential for hematopoiesis, immune function, vision, reproduction and embryonic patterning, as well as growth and differentiation in vertebrates (Sporn et al., 1994; Kastner et al., 1995; Chambon, 1996). However, both deficiency and excess of RAs and related retinoids during pregnancy cause abnormal morphological development (teratogenesis) of various organs and tissues, such as the eye, brain, heart, segment and limbs in the offspring (Collins and Mao, 1999; Zile, 2001). Teratogenesis by excess RAs has been observed in various vertebrates including fish (Herrmann, 1995; Haga et al., 2002), amphibians (Degitz et al., 2000, 2003; Alsop et al., 2004) and mammals (Lee et al., 1995; Ritchie et al., 2003). It has also been reported that high dietary intake of vitamin A by pregnant women can cause birth defects (Rothman et al., 1995). Based on the available evidence, it is clear that the environmental occurrence of RA mimics that disrupt RAR signaling may cause detrimental effects in animals and humans.

Gardiner et al. (2003) first detected RAR agonistic activity in the natural aquatic environment. During an investigation of the cause of the occurrence of deformed frogs in North America, they found that RARα agonistic activity was commonly found in water samples from a lake in Minnesota and a pond in California in which frog malformations were routinely found. However, the causative contaminants, which were named environmental retinoids, were not identified (Gardiner et al., 2003). Very recently, RAR agonistic activity was detected in wastewater treatment plants (WWTPs) and their receiving rivers in Beijing, China (Zhen et al., 2009) and several rivers in the Kinki region of Japan (Inoue et al., 2009). The results of these studies confirmed that RAR agonist contamination exists in the aquatic environment. In addition, the major causative RAR agonists in sewage in Beijing, China were first identified as 4-oxo-atRA and 4-oxo-13-*cis* RA (4-oxo-13cRA), which are oxidative metabolites of RAs, by Zhen et al. (2009). However, there is still very little available information regarding contamination of aquatic environments with RAR agonists. Specifically, to assess the possible risks of RAR agonists in the aquatic environment, it is still necessary to determine how widespread RAR agonist contamination is in the aquatic environment, how such contamination varies temporally and spatially, and whether 4-oxo-RAs are commonly the causative RAR agonists or other causative RAR agonists exist.

In this study, the RARα agonistic activity was determined in detail in the Lake Biwa–Yodo River and the Ina River in the Kinki region of Japan using a yeast two-hybrid assay to elucidate spatial and temporal variations in RARα agonist contamination and potential sources of RARα agonists in the rivers. In addition, bioassay-directed high performance liquid chromatography (HPLC) fractionation was conducted to characterize the causative contaminants.

2. Materials and methods

2.1. Chemicals

AtRA, 13cRA, 17β-estradiol (E2) and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 9cRA, dimethyl sulfoxide (DMSO) and ammonium acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile was purchased from Kanto Chemical (Tokyo, Japan). 4-Oxo-RAs (4-oxo-atRA, 4-oxo-9cRA and 4-oxo-13cRA) were purchased from Toronto Research Chemicals (Toronto, Canada). 2-Nitrophenyl-β-D-galactoside (ONPG) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Zymolyase-20T was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, USA). MeOH and acetonitrile were high-performance liquid chromatography (HPLC) grade, while the other chemicals were of the highest grade commercially available.

2.2. Surface water samples

Grab samples were collected from the subsurface zone (30- to 50-cm depth) at 20 stations along the Lake Biwa–Yodo River and 20 stations along the Ina River in the Kinki region of Japan (Fig. 1). The Lake Biwa–Yodo River is the largest watershed in the Kinki region of Japan, with a catchment area of 8,240 km^2. This river is the primary source of drinking water for the more than 14 million residents of Osaka City and its 24 circumjacent cities. The Ina River is a tributary of the Yodo River that runs along the border of Osaka and Hyogo Prefectures, with a catchment area of 383 km^2. The Ina River is a high priority river with respect to environmental conservation; however, this river is regarded as a highly polluted one as characterized with high levels of biochemical oxygen demand. Samples were collected at stations 2, 4, 8 and 18 of the Lake Biwa–Yodo River and stations 5, 8, 11, 12, 17 and 18 of the Ina River in June, August and October 2007 and January 2008. Samples were also collected from all 20 stations in the Ina River and Lake Biwa–Yodo River in April and October 2008, respectively. Additional samples were obtained from station 18 of the Ina River in July 2008 and April and October 2009. The quality of the surface water samples (range (median)) in the Lake Biwa–Yodo River and the Ina River were as follows: pH, 6.5–8.8 (7.2) and 6.4–8.7 (7.3); dissolved organic carbon, 1.3–6.7 mg/L (3.2 mg/L) and 0.37–12 mg/L (3.4 mg/L); total nitrogen, 0.092–5.8 mg/L (2.2 mg/L) and 0.41–14 mg/L (1.4 mg/L), respectively (Supplementary

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