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Detection of Agonistic Activities Against Five Human Nuclear Receptors in River Environments of Japan Using a Yeast Two-Hybrid Assay

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Abstract A total of 16 water samples from four rivers in Japan were examined for their agonistic activities against five human nuclear receptors (estrogen receptor [ER] α, thyroid hormone receptor α, retinoic acid receptor [RAR] α, retinoid X receptor α, and vitamin D receptor) by using a yeast two-hybrid assay. The results suggest that the river environment is contaminated with endocrine disrupting chemicals (EDCs) that can interact with a variety of nuclear receptors and that contamination with those that have RAR agonistic activity may be more serious than contamination with well-known EDCs that act as ER agonists.

Keywords Agonistic activity · Nuclear receptor · Retinoic acid receptor · River water environment

Endocrine disrupting chemicals (EDCs) are synthetic or natural compounds that disrupt or alter the functions of the endocrine system and consequently cause adverse effects in intact organisms (US EPA 1997). A major mechanism by which EDCs exert their potential disruptive effects on physiological processes is through their direct interaction with nuclear receptors (NRs). The occurrence of EDCs in natural aquatic environments has been studied worldwide especially focusing on steroid hormone receptors, such as the estrogen receptor (ER; e.g., Hohenblum et al. 2004; Quirós et al. 2004) on both compound- and activity-bases. However, recent research has revealed that the NR superfamily contains many different receptors in eukaryotic organisms (e.g., the human genome contains 48 NRs [Chawla et al. 2001]), each of which can mediate various adverse effects. Indeed, recent studies have shown that some environmental chemicals can potentially cause endocrine disruptive effects through interactions with a variety of NRs other than steroid hormone receptors (e.g., Nishikawa et al. 2003; Lemaire et al. 2005; Nakanishi et al. 2005). Therefore, a variety of NRs should be included in the assessment of potential endocrine disruptive effects in the aquatic environment. However, to date, the only reported investigations of agonistic activities against NRs in the aquatic environment have been on steroid hormone receptors, except for recent reports on studies targeting thyroid hormone receptor (TR; Murata and Yamauchi 2008) and retinoic acid receptor (RAR; Gardiner et al. 2003).

In this study, we examined the agonistic activities of surface waters collected from four rivers in Japan on five human NRs—ERα, TRα, RARα, retinoid X receptor (RXR) α, and vitamin D receptor (VDR)—by a yeast two-hybrid assay (Nishikawa et al. 1999) to assess whether river water might disrupt the endocrine functions mediated by these NRs. ER is a well-known steroid hormone receptor that plays a key role in female hormone regulation and signaling. Thyroid hormone signaling through TR is essential for cellular metabolism, growth, and the differentiation of many organs, including the brain. RAR and RXR are two NRs for retinoids (vitamin A and its metabolites); retinoid signaling is involved in vertebrate morphogenesis, growth, cellular differentiation, and tissue homeostasis. The VDR-dependent vitamin D endocrine system is central to the control of bone and calcium homeostasis.

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Materials and Methods

River water samples were collected from the subsurface zone (30–50 cm depth) of six stations along the Ina River in September 2006, three stations each along the Ibo River and Yomato River in October 2006, and four stations along the Yodo River in January 2007. The locations of the sampling stations and characteristics of the river water samples are shown in Fig. 1 and Table 1, respectively. All samples were transported on ice to the laboratory and subjected to physicochemical water quality measurements (dissolved organic carbon [DOC] and total nitrogen [T-N]) and solid phase extraction (SPE) within 12 h of the collection.

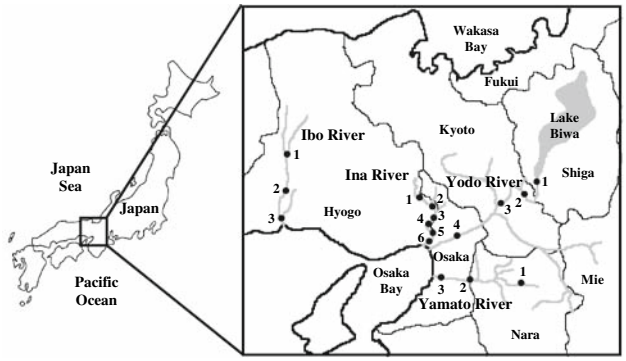

Fig. 1 Location of sampling stations

To avoid SPE cartridge plugging, suspended matter was removed by filtration through a Whatman GF/B filter (Whatman, Maidstone, Kent, UK). After filtration, 2 L of river water were extracted through an Oasis HLB cartridge (6 mL, 500 mg, Waters, Milford, MA, USA) previously conditioned with 6 mL of methanol and ultrapure water at a flow rate of 5–10 mL/min. The cartridge was washed with 6 mL of ultrapure water, and bound substances were eluted from the cartridge with 6 mL of methanol. The extracts were dried under a gentle nitrogen stream. The dry residue was dissolved in 200 μL of dimethyl sulfoxide (DMSO), resulting in a concentration factor of 10,000 fold as compared to the original river water. Prior to the assay testing the NR agonistic activity, the sample was diluted 10 and 100 times with DMSO (the resultant concentration factors were 1,000 and 100, respectively).

To evaluate the agonistic activity of the samples on NRs, we performed yeast two-hybrid assays using the recombinant yeast *Saccharomyces cerevisiae* Y190 into which one of the human NRs (ERα, TRα, RARα, RXRα, or VDR) and the coactivator TIF2 had been introduced (Nishikawa et al. 1999). Assays were carried out according to the procedure described by Nishikawa et al. (1999) with minor modifications. Briefly, aliquots of test samples (1% [v/v]) were incubated (30°C, 4 h) with the yeast cells pregrown (30°C, 18 h) in SD medium lacking tryptophan and leucine. Because the concentration factor of test samples applied were 100, 1,000 and 10,000, the final concentration factors of the test samples in the assay

Table 1 Characteristics of river water samples

River	Station	Distance (km)[a]	Sampling date	Temp (°C)	*p*H	DO[b] (mg/L)	DOC (mg/L)	T-N (mg/L)
Yodo River	1	0	Jan. 19, 2007	7.2	7.5	7.8	6.3	0.87
	2	21.2		6.5	7.6	8.1	5.5	0.80
	3	36.1		13.1	7.1	5.9	11	4.6
	4	57.7		8.4	7.5	4.2	6.1	1.7
Ina River	1	0	Sept. 3, 2006	25.4	7.5	5.3	2.2	NA[c]
	2	8.5		24.8	7.9	4.8	2.4	NA
	3	13.3		27.0	8.6	3.7	2.4	NA
	4	17.9		29.7	5.7	1.8	2.8	NA
	5	22.5		29.3	7.0	0.8	6.4	NA
	6	24.7		28.6	7.1	0.6	6.1	NA
Yamato River	1	0	Oct. 10, 2006	14.0	7.8	5.5	1.0	1.2
	2	30.0		21.8	7.6	4.2	1.9	3.7
	3	52.9		25.1	8.1	4.9	2.4	3.1
Ibo River	1	0	Oct. 10, 2006	14.6	6.7	9.7	11	0.57
	2	13.5		17.9	6.8	9.3	9.7	0.78
	3	25.9		18.0	6.9	9.4	10	0.83

[a] The distance from station 1 in each river is shown

[b] *DO* dissolved oxygen

[c] *NA* not analyzed

system were 1, 10, and 100, respectively. After measuring the yeast cell density (OD_{620}) with a plate reader (Viento XS, Dainippon Sumitomo Pharma, Osaka, Japan), the yeast cells were suspended in Z buffer containing 1 mg/mL Zymolyase-20T (ICN Biomedicals Inc., Costa Mesa, CA, USA) and digested at 30°C for 30 min. Then, 2-nitrophenyl-β-D-galactoside (Tokyo Chemical Industry, Tokyo, Japan) was added to initiate the β-galactosidase reaction. After a 20-min incubation at 30°C, Na_2CO_3 was added to stop the reaction, and the absorbance at 414 and 540 nm (A_{414} and A_{540}, respectively) was measured. The β-galactosidase activity was calculated as $1000 \times (A_{414} - 1.75 \times A_{540})/OD_{620}$. Negative control experiments were performed with 1% DMSO not containing any environmental sample, and 17β-estradiol (E2), 3,3′,5-triiodo-L-thyronine, all-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid, and 1α-25-dihydroxycholecalciferol were used as the positive controls for assays on ERα, TRα, RARα, RXRα, and VDR, respectively. All chemical compounds used were of the highest grade commercially available. All yeast two-hybrid assays were carried out in triplicate, except for the sample from station 1 of the Ina River for ERα agonistic activity, which was performed in duplicate due to limitation of the sample. Relative agonistic activity (%) was calculated as relative β-galactosidase activity to the maximal β-galactosidase activity given by the positive control for each NR (the concentration of positive control giving the maximal activity was 0.1, 1 or 10 μM, depending on the target NR). The relative agonistic activity of the negative control was set to 0%.

Data were analyzed with SPSS software (ver. 15.0 for Windows, SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to compare the agonistic activity of river water samples on each NR with that of the negative control. Differences were considered significant and highly significant at $p < 0.05$ and $p < 0.01$, respectively.

Results and Discussion

In almost all the cases in which significant activity was observed, the relative agonistic activity of a river water sample on each NR increased when the concentration factor was increased from 1 to 100, and no obvious inhibition by untargeted substances in the samples was observed (examples are shown in Fig. 2). We therefore evaluated the agonistic activity of the river water samples on each NR from the results at the concentration factor of 100.

Of the 16 samples tested, eight samples showed significant agonistic activity to ERα ($p < 0.05$), although the activity was at the most 1.1% of the maximal activity of the positive control (Fig. 3a). In the Yodo and Ina Rivers, the agonistic activity to ERα tended to increase at downstream

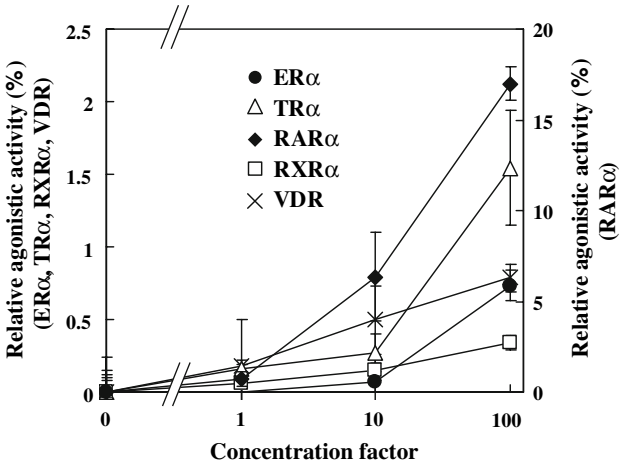

Fig. 2 Typical concentration-dependent NR agonistic activities of river water samples measured with a yeast two-hybrid assay. The results of a sample from station 6 in the Ina River are presented. The results for the negative control (DMSO only) are shown at the concentration factor of 0. Values are mean ± SD (n = 3). Results shown are normalized to the maximum activities of appropriate positive controls

stations. Significant TRα agonistic activity was detected in six samples (Fig. 3b). Similar to the findings with ERα, the activity increased at downstream stations in the Yodo (station 3) and Ina (station 5) Rivers. As shown in Fig. 3c, significant RARα agonistic activity was detected in all but 1 sample (from station 1 of the Yamato River). The relative agonistic activities on RARα were the highest among the five NRs tested, ranging from 2.7% to 24% of the highest agonistic activity of the positive control. Significant agonistic activities on RXRα and VDR were detected in six and nine samples, respectively (Fig. 3d, e). However, relative agonistic activities on these NRs were ≤0.4% and ≤0.9%, respectively, at a concentration factor of 100. In all five of the NRs tested, fewer samples had highly significant ($p < 0.01$) agonistic activity than significant ($p < 0.05$) activity: of the 16 samples, 6, 2, 10, 1, and 3 samples showed highly significant ($p < 0.01$) agonistic activity against ERα, TRα, RARα, RXRα, and VDR, respectively.

The maximal ERα agonistic activity was detected at station 5 in the Ina River. To estimate the contamination level by estrogenic compounds in the rivers analyzed here, we tentatively calculated the E2 equivalent from the dose-response curve of the sample. This revealed that the river water contained <0.3 ng/L (E2 equivalent) of estrogenic contaminants. The estrogenicity of river water samples used in this study appears to correspond to the very low (E2 equivalent of <0.1 ng/L) to high (0.1 to 1.7 ng/L) levels of estrogenic contamination reported by Quirós et al. (2004).

ERα agonistic activity increased at station 3 in the Yodo River and station 5 in the Ina River as compared with the corresponding upper stations (Fig. 3a, b). These stations

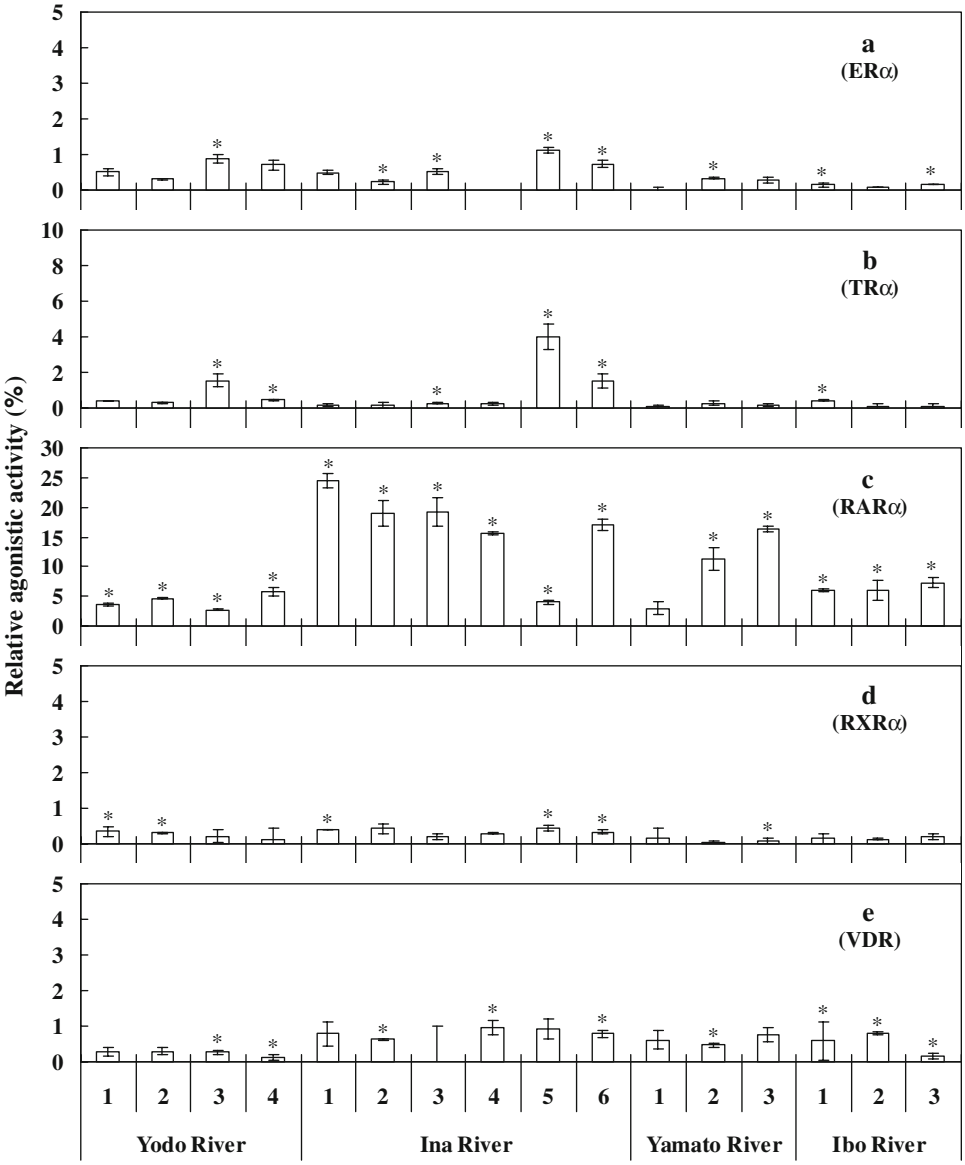

Fig. 3 Relative agonistic activities of river water samples against ERα (**a**), TRα (**b**), RARα (**c**), RXRα (**d**), and VDR (**e**). Agonistic activities at a concentration factor of 100 relative to the maximum obtained with the positive control are shown. Values are mean ± SD (n = 3, except for Ina River station 1, n = 2). *$p < 0.05$ versus the negative control, Student's *t*-test

are located downstream of sewage treatment plants (STPs). STPs are known to be a major source of natural estrogens such as estrone and E2, which are considered to be the main causes of estrogenic activity in the aquatic environment. Although these estrogenic compounds are mainly excreted in urine or feces in conjugated forms (i.e., as sulfates or glucuronides), the conjugates are cleaved during the biological treatment in STPs, thereby increasing the estrogenic activity (Ternes et al. 1999). Thus, the increase of ERα agonistic activity between stations 2 and 3 in the Yodo River and between stations 4 and 5 in the Ina River likely resulted from the inflow of effluent containing unconjugated natural estrogens from STPs.

The dramatic increase of agonistic activity on TRα at station 3 in the Yodo River and station 5 in the Ina River, similar to the increase of activity on ERα, suggested that STPs may be also the main source of EDCs that can disrupt thyroid hormone signaling via TR. Very recently, Murata and Yamauchi (2008) reported that municipal STP effluents contained contaminants with T_3-like activity. Although the causative compound(s) have not been identified yet, they are thought to be novel chemicals with no or extremely low polarity. Since methanol was used to condition the cartridges and elute the bound substances in the SPE step in this study, we expected to extract polar substances from the river water samples. Thus, the contaminant(s) with potential thyroid system-disrupting activity contained in our river water samples might be distinct from those detected in the previous study of Murata and Yamauchi (2008), suggesting that various kinds of thyroid system-disrupting contaminants may be present in the aquatic environment.

Almost all of the river water samples showed high agonistic activity on RARα (Fig. 3c). This may indicate that EDCs that can bind to RARα are widely present in the river environment. In addition, the trend of RARα agonistic activity along the watercourse was different from those of ERα and TRα: the relative agonistic activity on RARα was highest at station 1 and dramatically declined at station 4 (downstream of a large STP) in the Ina River; it also declined between stations 2 and 3 in the Yodo River. Considering the geological features of each sampling station, the main source of contaminants acting as RARα agonists may be not effluents from STPs but wastewaters from suburban and agricultural areas. Organochlorine pesticides have weak agonistic activity on RARβ and RARγ (Lemaire et al. 2005), and alkylphenols have weak agonistic activity on RARα, RARβ, and RARγ (Nishikawa et al. 2003). However, because we did not perform a chemical analysis, the contribution of these compounds and other contaminants to the RAR agonistic activities detected here is unclear.

Excess retinoid signaling can cause multiple developmental toxicities in various animals including humans (Rothman et al. 1995; Mulder et al. 2000; Degitz et al. 2003). To our knowledge, only Gardiner et al. (2003) have detected RAR agonistic activity in water samples, which they collected from a lake in Minnesota and a pond in California where frog malformations were routinely found; however, the causative contaminants, named as "environmental retinoids", were not identified. The ubiquitous detection of RARα agonistic activity in the river water environment in this study is therefore surprising.

The ATRA equivalent for the sample showing the highest RARα agonistic activity (station 1 in the Ina River) was calculated to be 4.5 ng/L. Degitz et al. (2003) reported that chronic (≥3 days) exposure to ATRA at ≥0.6 μg/L led to dysmorphogenesis of embryonic structures in *Xenopus laevis*. The detected RAR equivalent in the present study was two orders of magnitude lower than the ATRA concentration previously reported to cause RAR-mediated adverse effects, although this evidence does not always mean that negative effects by the putative environmental retinoids never occur in the freshwater environment we targeted.

Agonistic activities on RXRα and VDR were generally low for all of the river water samples tested. Organotin compounds such as tributyltin and triphenyltin have been identified as agonists of RXR (Nakanishi et al. 2005). Contamination by organotin compounds has been observed worldwide in the marine environment (Ueno et al. 2004), but similar contamination has not been observed in the freshwater environment. Thus, the low agonistic activities on RXRα observed in this study were not surprising.

In conclusion, we suggest that the river environment of Japan contains contaminants that could potentially disrupt endocrine systems through interactions not only with steroid hormone receptors but also with other NRs. In particular, contamination with environmental retinoids that act as RAR agonists may be ubiquitous in the river environment, and this is therefore one of the most important issues to address. The environmental retinoids present in the river environment should be identified to assess in detail the potential risks associated with altering retinoid signaling.

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