— **Review** —

Disruption of Retinoic Acid Receptor Signaling by Environmental Pollutants

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Retinoic acid (RA) receptors (RARs) are nuclear receptors that play a critical role in regulating cellular proliferation, development and differentiation in vertebrates in response to endogenous RAs, *i.e.*, all-*trans* RA and 9-*cis* RA. On the other hand, it has been well-known that both a deficiency and an excess of RA and related retinoids can cause a variety of teratogenic effects on developing embryos of vertebrates, which has been proven to be an RAR-mediated process. Therefore, the occurrence of xenobiotic environmental pollutants that interfere with RARs and disrupt the RAR signaling (*i.e.*, show RAR agonistic or antagonistic effects) may pose a threat to the health of wild animals and humans. This review mainly focuses on RAR agonists. We summarize the RAR agonistic activity of natural and xenobiotic compounds determined using *in vitro* bioassay systems and present recent field research showing the occurrence of RAR agonist contamination in the aquatic environment in North America, China and Japan. Environmental pollution by RAR agonists is a new endocrine disruption issue discovered very recently, and relevant knowledge is very limited. Further research will be required to obtain accurate information to assess the possible risks of RAR agonists in the environment.

Key words —— retinoic acid, retinoic acid receptor, retinoic acid receptor agonist, environmental pollutants, aquatic environment

INTRODUCTION

Vitamin A (retinol) and its biologically active derivatives (collectively referred to as retinoids), most notably retinoic acids (RAs), exert their pleiotropic effects on cellular proliferation, development and differentiation in vertebrates.[1–7] Furthermore, they have suppressive effects on carcinogenesis in various tissue types (*e.g.*, oral, skin, lung, liver, breast, bladder and prostate cancers).[8,9]

Retinoids elicit their effects through binding to two major families of retinoid-responsive nuclear receptors, RA receptors (RARs) and retinoid X receptors (RXRs).[5,6,10] Both RAR and RXR contain three isotypes, α, β and γ, with numerous isoforms.[5,6,8] RARs are activated by all-*trans* RA (atRA) and 9-*cis* RA (9cRA), while RXRs are activated only by 9cRA.[5,6,8] The RAR/RXR heterodimer, where RAR binds to a ligand and RXR does not, is the most common functional unit that transduces the retinoid signal at the gene level (Fig. 1).[11,12] In the basal state, the RAR/RXR heterodimer is bound to nuclear receptor corepressor or silencing mediator of retinoid and thyroid receptors.[13,14] Binding of the ligand leads to the conformational change of the complex and allows the release of corepressors and recruitment of coactivators, which results in the transcriptional activation of target genes via specific RA response elements (Fig. 1).[13,14] Well-known target genes of retinoid receptors are the *Hox* genes, a family of homeobox-containing genes having an essential role in the specification of positional information in the developing embryo.[16–18] By regulating the expression of *Hox* genes, the retinoid receptors largely affect the normal morphogenesis in vertebrates.

Contrary to the biological functions, it has been confirmed during last several decades that both a deficiency and an excess of RAs and related retinoids can cause teratogenic effects on various vertebrates. In addition, recent studies have

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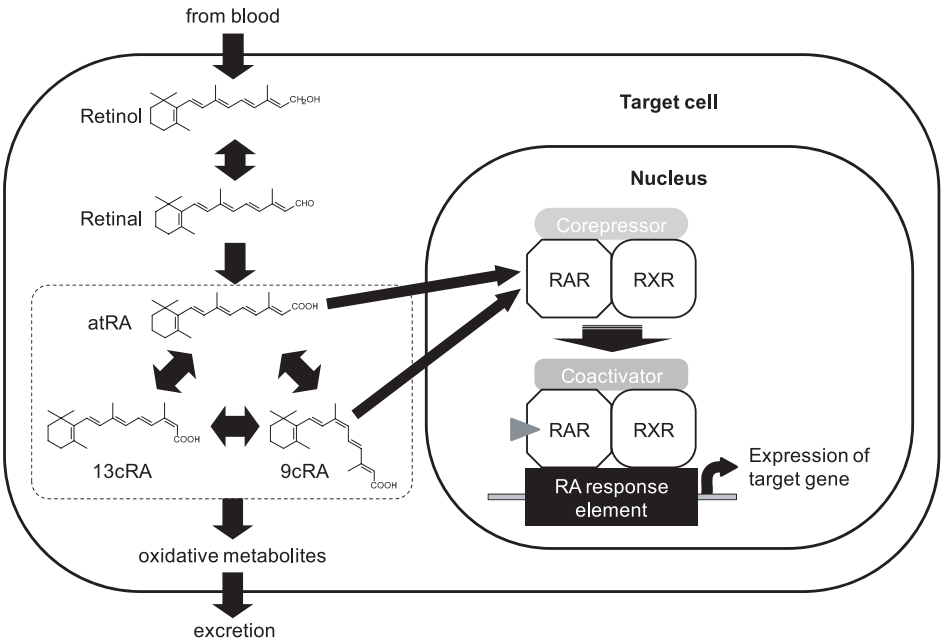

Fig. 1. Schematic Representation of the Metabolism of Retinoids and Activation of RAR Signaling in a Cell (Adapted from Refs. 13–15)).

shown that such RAR-mediated detrimental effects can occur or actually occur via environmental pollutants. Several reviews have recently summarized that some environmental pollutants (*e.g.*, polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and polycyclic aromatic hydrocarbons) can interfere with retinoid transport, metabolism and signaling in wild animals, and consequently cause adverse health effects.[14, 15, 19, 20] Furthermore, the presence of environmental pollutants, including those known and unknown, that have binding affinity to RAR and can directly disrupt RAR signaling through their RAR agonistic or antagonistic effects have been very recently confirmed by both laboratory experiments and field investigations. Based on the available evidence, the occurrence of RAR disrupting environmental pollutants has been recently recognized as a new endocrine disruption issue that results in detrimental effects on humans and wild animals.

This review focuses on the RAR agonists that can lead to excess RAR signaling. First, we make a brief summary concerning the biological effects on various animal species by excess RAR signaling. Then, we summarize the RAR agonistic potential of natural and xenobiotic compounds as determined in laboratory experiments and present recent field research that demonstrate the occurrence of RAR agonistic compounds in the aquatic environment.

BIOLOGICAL EFFECTS BY EXCESS RAR SIGNALING

While RAR signaling plays essential roles in the early development of vertebrates, ligands of RARs are also well-known potential teratogens in developing vertebrate embryos. Table 1 summarizes the abnormal morphological development in various animals induced by embryonic exposure to excess atRA, the natural and most potent RAR ligand. The type and degree of malformations induced differs depending largely on the species and timing (*i.e.*, developmental stage), duration and dosage of atRA administration. However, chronic exposure to atRA at concentrations equal to hundreds of nanograms a liter or more can cause a wide variety of dysmorphogenesis in various tissues (*e.g.*, eye, brain, limb and body axis) of fish, amphibians, birds, and mammals. Degitz *et al.*[26] reported that chronic exposure to atRA at as low as 600 ng/l for 3 d caused craniofacial deformities in *Xenopus laevis* embryos. In addition, it has been reported that ligands specific for each RAR isotype (α, β or γ) induce specific deformities. RARα ligands cause deformities of the ear, mandible and limb, RARβ ligands cause defects of the urinary system and liver, and RARγ ligands cause ossification deficiencies and defects of the sternebrae and vertebral body.[33–35]

Excess intake of retinoids can cause deleterious

Table 1. Teratogenic Effects Caused by Exposure to atRA in Various Vertebrates

Species	Dose (μg/l)	Exposure period	Observed biological effects	Ref.
Fish				
Zebrafish (*Danio/Brachydanio rerio*)	0.9	From stage 13 (onset of epiboly) to stage 23	Oedema and deformities of brain (reduction or absence of ventricle of the telencephalon) and tail (shortened and bent tail)	21)
	90	1 hr during late gastrulation (90–100% epiboly)	Multiple pectoral fins	22)
Japanese flounder (*Paralichthys olivaceus*)	3 (every day)	10 d from premetamorphic period	Fin deformity	23)
	7.5	6 to 9 d post-hatching	Deformities of lower jaw (growth retardation of the dentary), caudal fin (deformity of caudal bone complex and absence of entire caudal fin) and vertebrae (central fusion, hypertrophy of the centrum, and additional abdominal vertebrae)	24)
	15	40 hr from hatching period (2.5 d post-fertilization)	Deformities in mandible, hyoid and gill arches	25)
	30	1 hr at the shield stage (26 hr post-fertilization)	Deformity of lower jaw (absence of the Meckel's cartilage in mandible arch and fusion of cartilages in mandible and hyoid arches)	25)
Amphibian				
African clawed frogs (*Xenopus laevis*)	0.6	3 d from stage 8 to 41	Craniofacial deformity (microphthalmia, reductions in the prosencephalon and mesencephalon, and oedema)	26)
	6.25	24 hr from stage 8 (mid-blastula stage)	Microphthalmia and prosencephalic reduction	27)
	150	From stage 10 to 45 (mid-limb bud stage)	Loss of anterior structure	28)
	500	24 hr from stage 51 (mid-limb bud stage)	Hind-limb malformation	27)
Mink frog (*Rana septentrionalis*)	6.25	24 hr from stage 8 (mid-blastula stage)	Microphthalmia and prosencephalic reduction	27)
Green frog (*Rana clamitans*)	25	24 hr from stage 8 (mid-blastula stage)	Microphthalmia, prosencephalic reduction and posterior dysmorphogenesis	27)
Wood frog (*Rana sylvatica*)	500	24 hr from stage 28 (mid-limb bud stage)	Hind-limb malformation	27)
Bird				
White Leghorn (*Gallus gallus domesticus*)	Subblastodermal injection of 0.5 μg per embryo	HH stage 13 to early 14 (tail bud anlagen stage)	Caudal axial malformation	29)
Mammal				
Sprague-Dawley rat	10	46 hr from afternoon of 9 d post gestation	Deformities in the second visceral arch	30)
	150	48 hr from 9.5 d post coitum	Deformities in the branchial apparatus (hypoplasia of branchial arches and fusion of first and second branchial arches)	31)
Wistar-Imamichi rat	60	6 hr from 9 d post coitum	Reduction of the size of the first branchial arch, microcephaly, abnormal eye primordium, and open neural tube	32)
	60	6 hr from 9.5 d post coitum	Branchial arch fusion and open neural tube	32)

effects not only in wild animals but also in humans. Intake of large amounts of vitamin A (retinol) from supplements and use of RA congeners as therapeutic agents by pregnant women can increase the risk of birth defects on the central nervous system, ear, brain, heart, *etc*.[36, 37]

The available evidence on the biological effects resulted from the disruption of RAR signaling infers that the environmental occurrence of RA mimics that exhibit the RAR agonistic activity and disrupt the RAR signaling may cause detrimental effects in wild animals living in the polluted site. In addition, drinking water and eating food polluted with environmental RA mimics may increase the risk of disrupting the RAR signaling in humans that is normally caused by daily ingestion of vitamin A and RA congeners.

NATURAL AND XENOBIOTIC COMPOUNDS THAT ACT AS RAR AGONISTS

Besides RAs (natural RAR ligands), many endogenous compounds have been reported to be agonists of RAR. Oxidative metabolites of RA in humans and animals such as 4-oxo-RA, 4-hydroxy-RA, 18-hydroxy-RA and 5,6-epoxy-RA can bind to RARs and activate the RAR-mediated transcription via an RA response element.[28, 38–40] In particular, 4-oxo-RAs such as 4-oxo-atRA and 4-oxo-13-*cis* RA (4-oxo-13cRA) have been proven to have RAR agonistic activity equivalent to atRA using different *in vitro* bioassay systems.[28, 38, 40] Furthermore, numerous studies have demonstrated that these RA metabolites exhibit RAR-mediated biological activity just as RA does.[28, 41–45]

Recent studies have also revealed that xenobiotic environmental pollutants can elicit the binding affinity to and the agonistic activity on RARs. Xenobiotic compounds including not only well-known endocrine disrupting chemicals (EDCs) such as alkylphenols,[46–48] phthalate esters,[49] styrene dimers[48] and organochlorine pesticides[50] but also non-EDCs[51] have been proven to exhibit agonistic activity on one or several isotypes of RAR *in vitro* (Table 2). However, xenobiotic pollutants with agonistic activity at RARα are very limited as compared with those with RARβ or RARγ agonistic ability (Table 2). Kamata *et al*.[48] evaluated the RARγ agonistic activity of 543 compounds including industrial chemicals, agrochemicals, natural compounds, medicines and cosmetic chemicals with a yeast two-hybrid assay, and revealed that 85 chemicals including 16 organochlorine pesticides, 14 styrene dimers, 9 alkylphenols and 6 parabens were active at RARγ (showing $\geq 20\%$ of RARγ agonistic activity of 10 nM of atRA) at concentrations equal to 0.2–10 μM. Lemaire *et al*.[50] also reported that organochlorine pesticides having teratogenic activities in animals (*i.e.*, aldrin, chlordane, dieldrin, endrin and endosulfan) weakly activate RARβ and RARγ but do not activate RARα in a transactivation assay, and that among these five pesticides, endrin is the most effective at both RARβ and RARγ with 50% effective concentrations of 17.6 μM (6.7 mg/l) and 6.0 μM (2.3 mg/l), respectively. On the other hand, we revealed that telephthalic acid and linear dodecyl-benzensulfonate, both of which have not been regarded as EDCs, have binding affinity at RARγ.[51]

As shown in Table 2, RAR agonistic or binding activities of xenobiotic compounds reported to date are very low as compared with those of atRA. Therefore, these pollutants are not likely to disrupt RAR signaling and cause biological adverse effects at their normal environmental concentrations. However, some of xenobiotic compounds such as organochlorine pesticides are extremely persistent and tend to accumulate in biological tissues. Those compounds are further biomagnified in the food chain. Thus, despite their low RAR agonistic potencies, RAR-mediated biological effects might be possible if exposed to such a high level of xenobiotic compounds during the embryogenesis.

FINDINGS OF RAR AGONIST CONTAMINATION IN THE AQUATIC ENVIRONMENT

Despite the very low potency of currently known RAR agonistic environmental pollutants, several recent studies have detected a significant RAR agonistic activity in North America and China (Table 3). In some examples, adverse effects on aquatic animals probably caused by the RAR agonists that occur in the environment have also been observed.

Gardiner *et al*.[55] first detected RAR agonistic activity in the natural aquatic environment. They investigated the occurrence of RARα agonistic activity in two widely separated sites in the U.S.A. (a permanent lake in Minnesota and a vernal pond

Table 2. Representative Xenobiotic Compounds with RAR Binding/Agonistic Potency

Compound	RAR type	Binding/agonistic potency (assay system)[a)]	Ref.
4-Nonylphenol	RARα, β, γ	Weak activation (yeast two-hybrid assay)	46)
	RARγ	0.01–0.1% of atRA (CoA-BAP assay)	47)
	RARγ	0.476% of atRA (yeast two-hybrid assay)	48)
4-*t*-Octylphenol	RARα, β, γ	Weak activation (yeast two-hybrid assay)	46)
	RARγ	0.01–0.1% of atRA (CoA-BAP assay)	47)
	RARγ	0.997% of atRA (yeast two-hybrid assay)	48)
2-Chloro-4-octylphenol	RARγ	1.286% of atRA (yeast two-hybrid assay)	48)
2,6-Dichloro-4-octylphenol	RARγ	1.041% of atRA (yeast two-hybrid assay)	48)
4-*t*-Butylphenol	RARα, β, γ	Weak activation (yeast two-hybrid assay)	46)
2-*t*-Butylphenol	RARα, β, γ	Weak activation (yeast two-hybrid assay)	46)
4-*n*-Heptylphenol	RARγ	1.363% of atRA (yeast two-hybrid assay)	48)
Benzophenone	RARγ	0.1–1% of atRA (CoA-BAP assay)	47)
Hexachlorocyclohexane	RARγ	0.1–1% of atRA (CoA-BAP assay)	47)
	RARγ	0.668% of atRA (yeast two-hybrid assay)	48)
1-Phenyltetralin	RARγ	1.169% of atRA (yeast two-hybrid assay)	48)
Mono(2-ethylhexyl)phthalate	RAR from human prostate	Inhibition binding constant of 407 nM (competitive binding assay)	49)
Telephthalic acid	RARγ	0.001–0.1% of atRA (CoA-BAP assay)	51)
Linear dodecyl-benzensulfonate	RARγ	0.001–0.1% of atRA (CoA-BAP assay)	51)
Aldrin	RARβ, γ	Weak activation (transactivation assay)	50)
Chlordane	RARβ, γ	Weak activation (transactivation assay)	50)
Dieldrin	RARβ, γ	Weak activation (transactivation assay)	50)
Endrin	RARβ, γ	EC50[b)] of 17.6 ± 3.4 μM and 6.0 ± 0.8 μM for RARβ and RARγ, respectively (transactivation assay)	50)
Endosulfan	RARβ, γ	Weak activation (transactivation assay)	50)

a) RAR binding/agonistic potencies of xenobiotic compounds were calculated based on the lowest effective concentration in CoA-BAP (coactivator-bacterial alkaline phosphatase) assay[47,51] and also based on the 20% relative effective concentration in yeast two-hybrid assay.[48] *b*) 50% Effective concentration. The 100% value was obtained in the presence of 10 nM of (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB), a synthetic RAR agonist that activates all isotypes of RAR with equivalent potency to atRA.[52–54]

Table 3. RAR Agonist Contamination Detected in Different Countries

Country	Location or sample	Causative compounds	Ref.
U.S.A.	• A permanent lake in Minnesota and a vernal pond in California, where malformed frogs were frequently observed	Unidentified	55)
Canada	• Effluent from 11 pulp mills	Unidentified	39)
China	• Influent and effluent from 7 wastewater treatment plants (WWTPs) receiving mainly domestic wastewater	4-Oxo-atRA and 4-oxo-13cRA	40)
	• Surface water from Tonghui River and Qing River in Beijing		
Japan	• Surface water from 4 rivers in the Kinki region	Unidentified	56, 57)
	• Influent and effluent from 7 municipal WWTPs in Osaka Prefecture and 3 municipal WWTPs in Toyama Prefecture	Unidentified	58, 59)

in California) where deformed frogs had been frequently discovered. Consequently, a high response of RARα agonistic activity was detected commonly in both sites.[55] From the results in this study and another study, Gardiner and co-workers suggest that the occurrence of environmental pollutant(s) acting as RAR agonists, which were named "environmental retinoid(s)," is the most probable cause of the outbreak of deformed frogs that occurred in the upper Midwest U.S.A. and Canada.[55,60]

In another study by Alsop *et al.*,[39] to clarify the cause of dramatic reductions of stored hepatic retinoids observed in adult male and female white suckers (*Catostomus commersoni*) in the Mattagami

River in northeastern Ontario (Canada) receiving the bleached kraft mill effluent, the methanol and dichloromethane extracts from the effluent of pulp mills across Canada were examined for their binding to rainbow trout (*Oncorhynchus mykiss*) RARs from the gill. Competitive binding assays from their study demonstrated that the final effluent from several tested mills exhibited the ability to displace [^{3}H]atRA from the gill RAR in a dose-dependent manner.

A few years after the findings that reported the occurrence of RAR agonists in North America, a detailed survey on RAR agonist contamination was performed in Beijing, China by our research group because adverse effects such as curved tail tip and reduction of hatching success rate had been observed on Japanese medaka (*Oryzias latipes*) embryos exposed to the secondary effluent from a wastewater treatment plant (WWTP). We investigated the RARα agonistic activity in seven WWTPs and their receiving rivers (Tonghui River and Qing River) and detected a significant activity mainly in the ethyl acetate fraction (medium polar fraction) of the wastewater and water samples.[40] The maximal atRA equivalents (atRA-EQ) in the fraction estimated based on the results of yeast two-hybrid assay were 13.4, 3.2 and 10.0 ng/l in WWTP influent, WWTP effluent and river water, respectively, which were much lower than the atRA concentration exhibiting teratogenic effects in animals (Table 1). Most of the RARα agonistic activity detected in river water seemed to be explained by WWTP effluents and untreated wastewater discharged from several wastewater discharging pipes. However, unexpectedly high activities obtained in the upstream of the Qing River suggested the presence of unknown significant sources.

Causative compounds for the RAR agonist contamination have not been identified in the studies performed in North America (Table 3).[39,55] However, results in these studies suggest the presence of multiple RAR agonists in aquatic samples.[39,55] In addition, Gardiner *et al*.[55] suggest from the characteristics of their study sites that the causative RAR agonists for frog malformations are not natural in origin. By contrast, our study in Beijing, China succeeded in identifying the major RAR agonists present in sewage as 4-oxo-atRA and 4-oxo-13cRA (Table 3).[40] 4-Oxo-atRA and 4-oxo-13cRA are generated in human and animal bodies by the metabolism of RAs and are eliminated from the bodies through urinary excretion mainly as glucuronide conjugates (retinoyl-β-glucuronides).[13,34,61,62] Thus, it is likely that 4-oxo-atRA and 4-oxo-13cRA are reproduced through the deconjugation of their glucuronides in the sewage system and WWTPs. Because the concentrations of both 4-oxo-RAs were largely reduced from influent to effluent irrespective of the WWTP,[40] these compounds appear to be easily removable from the water phase through the degradation by activated sludge microorganisms and/or the adsorption onto activated sludge flocs in WWTPs. In addition, we also suggested the presence of other unidentified RAR agonists in the river water because 4-oxo-RAs could not account for the total RAR agonistic activity in samples upstream from the WWTP on the Qing River.[40] Based on the currently available evidence, multiple unknown RAR agonists seem to exist in the aquatic environment.

ENVIRONMENTAL POLLUTION BY RAR AGONISTS IN JAPAN

Environmental pollution by RAR agonists occurs not only in foreign countries but also in Japan (Table 3). Our recent studies in 4 rivers in the Kinki region and in several WWTPs in Osaka and Toyama Prefectures detected statistically significant RARα agonistic activity commonly in surface waters in all of the investigated rivers[56,57] and influents and effluents from the WWTPs.[58,59] River water pollution by RAR agonists occurs irrespective of the pollution level of the rivers, and surprisingly even in the upstream suburban areas with little human activity.[56,57] In addition, strong RARα agonistic activity unaccountable by the activity of WWTP effluents was detected in river water samples.[56,57,59] Therefore, WWTPs do not seem to be the major source of RAR agonists in our study areas in Japan, which is different from the RAR agonist pollution found in Beijing, China as described above. Also, simultaneous investigation of the agonistic activities at RARα and estrogen receptor α suggests that the variation along the watercourse of RAR agonist pollution in Japanese rivers is completely dissimilar to that of the estrogenic contamination, a widely known EDCs problem.[56,57] Although the RAR agonists present in aquatic samples in Japan are not yet identified (Table 3), the river pollution level in the Kinki region of Japan appears to be greater than that in Beijing, China.[40,57]

However, the maximal atRA-EQ in river water was estimated to be 47.6 ng/l,[57] which was more than 10-fold lower than the atRA level leading to the abnormal morphological developments in vertebrates (Table 1). Therefore, the level of RAR agonist pollution is not likely to cause RAR-mediated deleterious biological effects at present.

CONCLUSIONS

In this review, we have presented the recent evidence regarding RAR agonist contamination in the aquatic environment. Although the potency of known RAR agonistic xenobiotic compounds is very low, pollution of the aquatic environment by RAR agonists has been recently observed in widely separated countries (North America, China and Japan). These findings suggest that the environmental pollution by RAR agonists occurs widely in the aquatic environment. In addition, the study in North America[55] demonstrates that unidentified RAR agonists present in the environment can really threaten the health of wild animals by disrupting their RAR signaling. Therefore, further studies, particularly the identification of unknown RAR agonists and the investigation of the occurrence and fates of both already-known and unknown RAR agonists present in the aquatic environment, are needed to understand the overall picture of the environmental pollution with RAR agonists. Studies concerning the toxicity of those RAR agonists occurred in the aquatic environment, *e.g.* determination of their agonistic activities on RARs of different animal species and evaluation of the correlation between the RAR agonistic activity obtained from *in vitro* bioassays and the toxicity *in vivo*, should be also carried out to assess the possible ecological risks resulted from the RAR agonist pollution.

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