Aquatic Toxicology 199 (2018) 103–115

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Characterization and comparison of transcriptional activities of the retinoid X receptors by various organotin compounds in three prosobranch gastropods; *Thais clavigera, Nucella lapillus* and *Babylonia japonica*

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ARTICLE INFO

Keywords:
Thais clavigera
Babylonia japonica
Nucella lapillus
Retinoid X receptor (RXR)
Imposex
Organotins

ABSTRACT

Two cDNAs of RXR were isolated, for the first time, from the ivory shell, *Babylonia japonica*, and the transcriptional activities were tested *in vitro* to compare with other gastropod (*Thais clavigera* and *Nucella lapillus*) RXR isoforms. The transcriptional activities of all of these RXR isoforms were significantly induced by mammalian RXR agonist, 9-*cis* retinoic acid (9cRA). The transcriptional activity of *T. clavigera* RXR-1 was also examined by using 9cRA and 16 organotin compounds, and significant ligand-dependent transactivations were observed by 9cRA and 5 organotins (tributyltin (TBT), tetrabutyltin (TeBT), tripropyltin (TPrT), tricyclohexyltin (TcHT) and triphenyltin (TPhT)). These 5 organotins also induced significant transcriptional activities in *N. lapillus* and *B. japonica* RXR isoforms. These 4 organotins, except for TeBT, have been reported to promote the development of imposex after a month of a single injection each, using female *T. clavigera*. To investigate the function of gastropod RXR isoforms, the effects of mammalian specific pan-agonist, PA024, and pan-antagonist, HX531, were examined, and significant induction of transcriptional activity by PA024 was demonstrated in these gastropod RXR isoforms. The additions of HX531 significantly suppressed the transcriptional activities of these gastropod RXR isoforms by 9cRA and 5 organotins. Using the mammalian two retinoic acid response elements, the transcriptional activities by 2 agonists, 9cRA and PA024, were different among the RXR isoforms of each gastropod species. With retinoid X response element (RXRE), transcriptional activities of TcRXR-1, BjRXR-1, and NlRXRa were significantly higher than those of TcRXR-2, BjRXR-2, and NlRXRb. Transcriptional activities of TcRXR-2, BjRXR-2, and NlRXRb, however, were significantly higher than those of TcRXR-1, BjRXR-1, and NlRXRa with thyroid hormone response element, TREpal. Thus, induction of imposex in prosobranch gastropods is strongly suggested to be triggered by 9cRA and certain organotins, such as TBT and TPhT through the activation of RXRs. These gastropod RXRs might control the different gene transcription by forming homo- or heterodimer complex with their own isoforms. These findings will contribute to our understanding of the fundamentals of the endocrine system in molluscs, particularly on RXR signaling pathway.

1. Introduction

Aquatic environmental pollution by organotin compounds has been spread worldwide because organotins, such as tributyltin (TBT) and triphenyltin (TPhT), have been extensively used in antifouling paints for ships and fishing nets since the mid-1960s (Bennett, 1996; Bryan et al., 1986; Hoch, 2001; Horiguchi et al., 1994). Meanwhile, organotins are known to have various harmful effects on both vertebrates and

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https://doi.org/10.1016/j.aquatox.2018.03.029
Received 25 December 2017; Received in revised form 21 March 2018; Accepted 23 March 2018
Available online 26 March 2018
0166-445X/

invertebrates (Boyer, 1989; Fent, 1996; Golub and Doherty, 2004). The induction of 'imposex' in female gastropod molluscs is one of the most well-known adverse effects caused by organotins (Bryan et al., 1986, 1987, 1988; Gibbs et al., 1987, 1988; Horiguchi et al., 1994, 1995, 1997a; Smith, 1971, 1980, 1981a,b,c,d). The term 'imposex' has been defined as a superimposition of male genital organs, such as the penis and vas deferens, on female prosobranch gastropods, which is considered an irreversible pseudohermaphroditic condition, resulting in population decline or mass extinction due to reproductive failure of females at severely polluted sites (Bryan et al., 1986; Gibbs and Bryan, 1986, Gibbs et al., 1988, 1990; Horiguchi and Shimizu, 1992; Horiguchi et al., 1994, 1997b, 2006; Smith, 1971). Approximately 200 species of Caenogastropoda have been described to be affected by imposex (Bech, 2002a,b; Fioroni et al., 1991; Horiguchi, 2000; Horiguchi et al., 1997b; Marshall and Rajkumar, 2003; Matthiessen et al., 1999; Shi et al., 2005; Solé et al., 1998; Ten Hallers-Tjabbes et al., 2003; Terlizzi et al., 2004). Environmental monitoring surveys on contamination by organotins and imposex in prosobranch gastropods have been conducted worldwide, including Africa, and Central and South America regions (Caetano and Absalão, 2002; Castro et al., 2007a; Domínguez-Ojeda et al., 2015; Elhasni et al., 2013; El Mortaji et al., 2011; Lahbib et al., 2008, 2009; Lopes-dos-Santos et al., 2014; Teso and Penchaszadeh, 2009; Vasconcelos et al., 2010). Moreover, efficiency of domestic or regional legislations on TBT-based antifouling paints, as well as the International Convention on the Control of Harmful Antifouling Systems on Ships (known as AFS Convention), which came into force on 17 September 2008, has been assessed, on the basis of results on those monitoring surveys (Galante-Oliveira et al., 2011; Laranjeiro et al., 2015, 2018).

The chemical species of organotin compounds which can induce and/or promote the development of imposex varies slightly among gastropod species: for example, TBT can induce and promote the development of imposex in the dog-whelk, *Nucella lapillus*, but contrasting results have been reported regarding the ability of TPhT to promote the development of imposex in *N. lapillus*, with some studies pointing to a non-significant effect (Bryan et al., 1987, 1988; Gibbs et al., 1987) or a significant effect (Laranjeiro et al., 2016). Both TBT and TPhT, however, are known to induce and promote the development of imposex in the rock shell, *T. clavigera* (Horiguchi et al., 1995, 1997a, 2010, 2012). The induction of imposex by TPhT has also been reported in a few species other than *T. clavigera* (Barroso et al., 2002, Schulte-Oehlmann et al., 2000, Santos et al., 2006). It also seems there is a difference in the developmental order of vas deferens as well as penis in imposex-exhibiting females even among Muricidae species, especially in the initial developmental stages of imposex, on the basis of the vas deferens sequence indices in *N. lapillus* and *T. clavigera* (Gibbs et al., 1987; Horiguchi et al., 2012). In the ivory shell, *B. japonica* (Buccinidae), the development of vas deferens and penis in imposex-exhibiting females also seems to differ from that in *T. clavigera* (Horiguchi et al., 2006, 2012, 2014). Thus, there could be a difference in chemical species of organotins responsible for inducing and promoting the development of imposex and the developmental order of vas deferens and penis in imposex-exhibiting females among gastropod species.

In *T. clavigera*, a single injection of either 9-*cis* retinoic acid (9cRA) or TPhT into females induces the development of imposex after a month, suggesting that imposex is induced through the activation of retinoid X receptor (RXR) (Nishikawa et al., 2004). Horiguchi et al. (2010) investigated the time course of expression of the RXR gene in various tissues (ctenidium, ovary or testis, digestive gland, penis-forming area or penis, and head ganglia) of female and male *T. clavigera* exposed to TPhT in a flow-through exposure system for 3 months. In females, 3-month exposure to TPhT resulted in the development of imposex, and RXR gene expression in the ovary, penis-forming area or penis, and head ganglia of females exposed for 3 months was significantly higher than expression in control females. The highest RXR gene expression was found in the penis-forming area or penis, and moreover, RXR gene expression in the penis-forming area or penis of each female exposed to TPhT seemed to be associated with an increase in penis length. In *N. lapillus*, a similar phenomenon was observed by Lima et al. (2011). They reported that a significant ($P \leq 0.001$) increase in transcription of RXR gene was observed in penis of females with vas deference sequence index (VDS) levels of 3–4 in comparison with the penis-forming area of both control and imposex females with VDS 1–2 after the imposex induction. At advanced stages of imposex, females displayed RXR transcription patterns in penis identical to those of males (Lima et al., 2011). Thus, in gastropods, RXR could play an important role in the development of male genitalia (i.e., penis and vas deferens), although RXR might also have other physiological functions. These observations strongly suggest that the activation of RXR could be involved in inducing and promoting the development of imposex, namely the development of male genitalia (i.e., penis and vas deferens) in female prosobranch gastropods. RXR isoforms have been also reported in a few species of gastropods (Castro et al., 2007b, Nishikawa et al., 2004, Urushitani et al., 2011), and a bivalve (Lv et al., 2013) among Mollusca.

Meanwhile, previous studies have also revealed that TBT and TPhT function as ligands that activate not only the transcription of molluscan RXRs, but also of mammalian and crustacean RXRs (Nishikawa et al., 2004; Wang and LeBlanc, 2009). In vertebrates, three RXR isoforms (α, β and γ) form homodimer itself or heterodimers with other nuclear receptors, such as retinoic acid receptor, vitamin D receptor, thyroid hormone receptor, peroxisome proliferator-activated receptor, liver-X receptor, bile acid receptor, and xenobiotic compound receptor (Wolf, 2006). These dimers bind to specific DNA sequence, known as retinoic acid response elements, in the genomic promoter region. These elements consist of direct (DR), inverted (IR), everted (ER), or palindromic (pal) repeated half-site DNA sequences (Dawson and Xia, 2012). In human RXR subtypes, these genes are located on three different chromosomes, and their sequences and functions are independent from each other (Dawson and Xia, 2012). The molluscan RXR isoforms, however, only differ in some amino acid residues in the T-box of the DNA binding domain (C domain) (Urushitani et al., 2011; Lv et al., 2013). In Chinese scallop (*Chlamys farreri*), these isoforms are generated via alternative mRNA splicing (Lv et al., 2013). This insertion of molluscan-like amino acid residues in T-box of RXR has also been reported in several crustaceans (Kim et al., 2005; Priya et al., 2009; Wu et al., 2004), and the ecdysteroid receptor (EcR) forms heterodimers with crustacean RXR (Durica et al., 2002; Hopkins et al., 2008). In molluscs, the candidate partners for RXR heterodimerization have been reported in *Lottia gigantea* (Albalat and Cañestro, 2009); however, it is unknown whether these receptors could be functioning as heterodimer with other molluscan RXRs.

In the present study we isolated, for the first time, two cDNA clones encoding RXR isoforms from *B. japonica* with the purpose to investigate the relationship between RXR pathway and the development of imposex by organotins, and we performed transactivation assays using three RXR isoforms of gastropod species (*T. clavigera*, *N. lapillus* and *B. japonica*). Moreover, we also studied the transcriptional activities of these gastropod RXR isoforms by using direct or inverted repeats of half-site retinoic acid response elements to investigate the function of RXR isoforms.

2. Materials and methods

2.1. Animals and chemical reagents

B. japonica were purchased and transported to the National Institute for Environmental Studies (NIES) from the Yodoe fisherman's cooperative association in Tottori prefecture, Japan in April 2010. *N. lapillus* were collected in a non-polluted location at Poço da Cruz, Mira, Portugal in October 2010. The snail specimens were dissected immediately after collection and preserved in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA) for RNA extraction. These