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RESEARCH ARTICLE

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Imposex in marine gastropods may be caused by binding of organotins to retinoid X receptor

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Abstract Organotin compounds have been widely used as antifouling paints for ships and fishing nets since the 1960s and have thus been released into marine environments. Aquatic invertebrates, particularly marine gastropods, are extremely sensitive to organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) and undergo changes in sexual identity in response to exposure. This worldwide phenomenon is one of the worst consequences of pollution by man-made chemicals and has led to the ban of such compounds in antifouling paints in a number of countries, although organotin compounds still exist in the environment. So far, very low-concentrations of TBT or TPT have been shown to induce imposex (superimposition of male genitalia on female) in marine gastropods. Although the imposex induction mechanism has been controversial for many years, it was recently reported that TBT and TPT are potent and efficacious activators of retinoid X receptor (RXR), a member of the nuclear receptor superfamily. In this review, I discuss the involvement of RXR in the development of gastropod imposex.

Introduction

In their book "Our Stolen Future", Colborn et al. (1996) pointed out that a number of environmental chemicals affect hormonal systems and have adverse health effects on wildlife and probably on humans. Such chemicals are referred to as endocrine disruptors, and their effects have emerged as a major environmental issue. The nuclear receptors of intrinsic hormone systems are likely to be targets of endocrine disruptors, because their intrinsic ligands are fat-soluble and low-molecular-weight agents, as are the environmental pollutants. Many synthetic compounds, including the drug diethylstilbestrol (DES), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCB), and alkylphenols, have been shown to bind nuclear receptors (Sohoni and Sumpter 1998; Blair et al. 2000; Nishihara et al. 2000; Gray et al. 2001). The effects of synthetic chemicals on sex hormone receptors such as the estrogen receptor (ER) and androgen receptor (AR) have attracted much attention, focusing on the reproductive failures observed in wildlife.

Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s. Their release into the marine environment has resulted in pollution worldwide. Most marine gastropods in organotin-polluted areas have shown reproductive failure due to oviduct blockage by vas deferens formation, resulting in population decline or mass extinction (Bryan et al. 1986; ten Hallers-Tjabbes et al. 1994). This phenomenon is called "imposex" as an abbreviation of "imposed sexual organs", because male genital organs, such as the penis and vas deferens, are imposed upon female organs (Smith 1971). Approximately 150 species of imposex-affected gastropods have been found in the world (Fent 1996; Matthiessen et al. 1999). Gastropod imposex is reportedly induced by very low concentrations of TBT or TPT and is thought to be one of the mechanisms of endocrine disruption in wildlife (Smith 1971; Bryan et al. 1986, 1987, 1988; Gibbs and Bryan 1986; Gibbs et al. 1987; Axiak et al. 1995; Horiguchi et al. 1997b). Despite several hypotheses on the cause of imposex induction, such as aromatase inhibition, testosterone excretion-inhibition, functional disorder of the female cerebropleural ganglia, and involvement of amidated tetrapeptide Ala-Pro-Gly-Trp-NH2 (APGWamide) (Bettin et al. 1996; Ronis and

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Mason 1996; Oberdörster and McClellan-Green 2000, 2002), the detailed biochemical mechanism behind this phenomenon remains obscure.

It is well known that steroidal sex hormones such as 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT) exert important roles in physiological processes, including sexual development and reproduction in vertebrates. However, homologues of ER and AR have not been found in invertebrates (Escriva et al. 1997). Because gastropods are mollusks, they may not have functional receptors for androgen, suggesting that vertebrate-type sex hormones may not be involved in male sexual development in the gastropods. Recently, it was reported that TBT and TPT are high-affinity ligands for human retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) γ (Kanayama et al. 2005). In addition, a functional homologue of RXR has been cloned from the rock shell (*Thais clavigera*) and the natural ligand of RXR, 9-*cis* retinoic acid, induces imposex in this species (Nishikawa et al. 2004). These reports suggest that the induction of imposex by organotin compounds may be mediated by RXR.

Differences in nuclear receptors between invertebrates and vertebrates

Nuclear receptors are structurally related proteins classified into a large superfamily that includes receptors for hydrophobic molecules such as steroid hormones (e.g., estrogens, androgens, progesterone, glucocorticoids, mineralocorticoids), retinoic acids (all-*trans* and 9-*cis* isomers), thyroid hormone, 1,25 $(OH)_2$ vitamin D_3, fatty acids. In addition to these receptors, the superfamily also contains a large number of so-called orphan nuclear receptors whose ligands do not exist or have not been identified (Giguère 1999). Nuclear receptors share a common structural organization with a highly conserved DNA-binding domain and a moderately well-conserved ligand-binding domain (LBD) (Fig. 1). Phylogenetic study and extensive polymerase chain reaction (PCR) surveys have revealed that nuclear receptor genes appeared very early on during metazoan evolution, but could not be found in fungi, plants, or unicellular eukaryotes (Escriva et al. 1997, 2000). By virtue of genome projects, we now know that *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, respectively, have 48, 21, and 220 kinds of nuclear receptor genes (Maglich et al. 2001). There is a striking difference between vertebrates and invertebrates with respect to their nuclear receptor sets. For instance, receptors for sex and adrenal steroid hormones have not been found in any fully sequenced invertebrate genomes. Although ER-like cDNA was reportedly isolated from the mollusk *Aplysia californica*, it could not bind to estrogens and was a constitutive activated transcription factor like the orphan nuclear receptors (Thornton et al. 2003). So far, functional steroid hormone receptors including AR, ER, progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), have not been found in any invertebrate species (Escriva et al. 1997; Laudet 1997).

Reproductive abnormalities in wildlife can be associated with exposure to environmental pollutants capable of mimicking the action of sex hormones. In fact, there are many synthetic chemicals that have been shown to possess estrogenic activity by in vitro binding assay, reporter gene assay, or uterotrophic assay. The typical characteristic of chemicals having estrogenic activity is a phenol with a hydrophobic moiety at the para-position and without bulky groups at the ortho-position (Blair et al. 2000; Nishihara et al. 2000). Although these compounds may have adverse health effects in vertebrates (Colborn et al. 1996), they may not alter the function of the reproductive system through the medium of ER in invertebrates.

Imposex in marine gastropods

Among the variety of endocrine-disrupting events in marine invertebrates, imposex is one of the most documented. Imposex is induced by TBT at concentrations as low as 1 ng/L of tin (Sn) (Gibbs et al. 1987; Axiak et al. 1995) and is used extensively all over the world as a biomarker to monitor TBT pollution (Gibbs et al. 1987; ten Hallers-Tjabbes et al. 1994; Horiguchi et al. 1997a; Terlizzi et al. 1998, 2004). Not only TBT but also TPT has been shown to have a strong effect on the development of imposex in *T. clavigera* (Horiguchi et al. 1997b). So far, several hypotheses have been proposed to explain imposex induction. The first is that TBT increases androgen levels by inhibiting the enzyme activity that metabolizes testosterone. An aromatase enzyme complex is responsible for converting androgenic to estrogenic steroids. This enzyme complex consists of the microsomal CYP19 enzyme and the flavoprotein nicotinamide adenine dinucleotide phosphate reduced-form reductase. The latter is responsible for transferring reducing equivalents to CYP19 within the membrane of the endoplasmic reticulum. Bettin et al. (1996) reported that TBT increases androgen levels through inhibition of aromatase activity in marine neogastropods at relatively high doses. The TBT also inhibits the catalytic activity of human aromatase from transfected cells or a granulosa cell-like tumor cell line (Cooke 2002; Heidrich et al. 2001; Saitoh et al. 2001). However, it is doubtful whether the inhibitory effect of TBT on aromatase activity is a cause of the imposex, because the role of vertebrate sex steroids is unclear in invertebrates (LeBlanc et al. 1999). The second hypothesis is that TBT acts as a neurotoxin to abnormally release the peptide hormone termed penis morphogenic factor (PMF) (Féral and Le Gall 1983). The peptide hormone APGWamide has been proposed as the putative PMF, because injection of APGWamide significantly induces imposex in the mud snail *Ilyanassa obsoleta* (Oberdörster and McClellan-Green 2000,

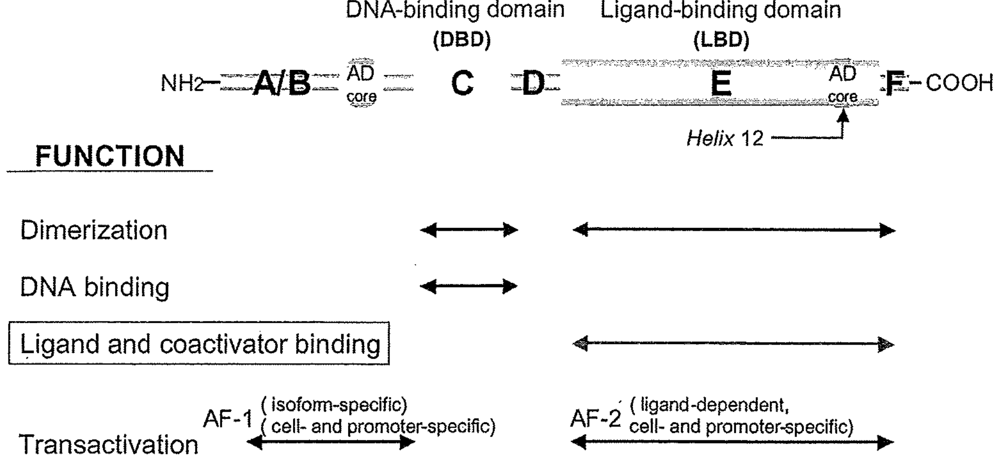

Fig. 1 Typical structure and functional domain of a nuclear receptor. Nuclear receptors are highly structurally related and share a common structural organization with a variable amino-terminal domain (**a/b**); a central, well-conserved DNA-binding domain (**c**); a non-conserved hinge domain (**d**); and a carboxyl-terminal, moderately conserved ligand binding domain (**e**). The ligand-independent transactivation function (**af-1**) is contained within the **a/b** region, and the ligand-dependent transactivation function (**af-2**) is within the **e** region

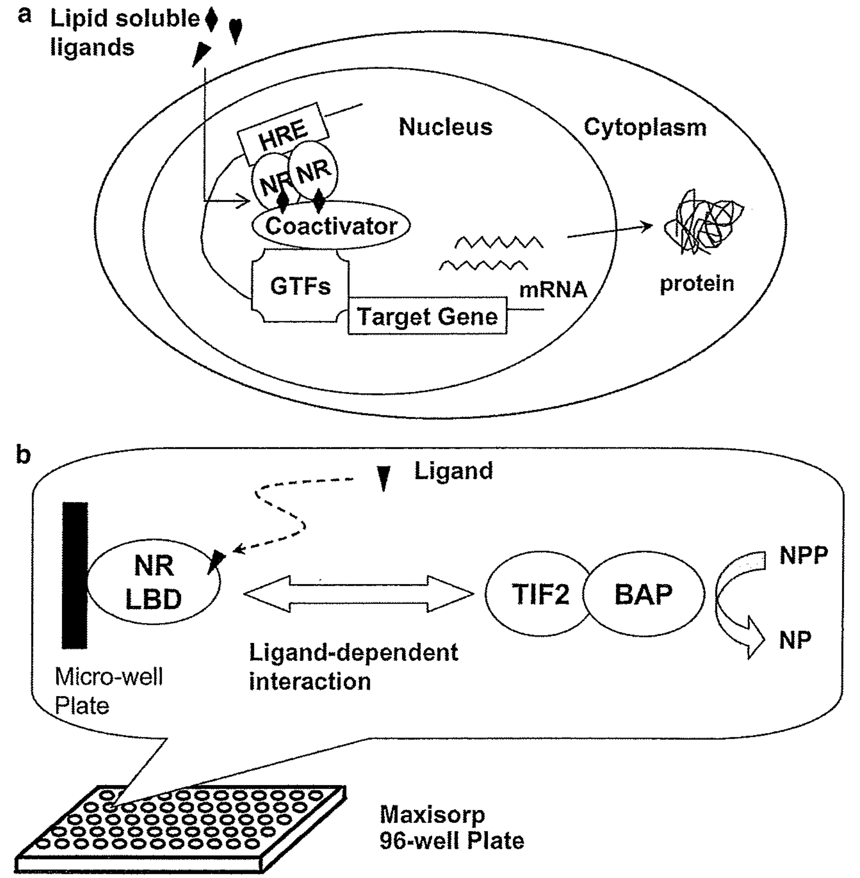

Fig. 2 **a** Nuclear receptors act as ligand-activated transcription factors by directly interacting with DNA-response elements of target genes as homodimers, heterodimers, or monomers. The effects of nuclear receptors on transcription are mediated through recruitment of co-regulators. Upon ligand binding, the receptors undergo a conformational change that allows the recruitment of coactivator complex. Recruitment of coactivator complex to the target promoter causes chromatin decomposition and transcriptional activation through interaction with general transcription factors (*GTFs*). **b** Principle of the screening method for nuclear receptor ligand. Nuclear receptor ligand-binding domain (*NRLBD*) is immobilized on the surface of a 96-well microplate. Coactivator TIF2 is prepared as a fusion protein with bacterial alkaline phosphatase (*BAP*). Test chemicals are added to the well with TIF2-BAP fusion protein. If the test chemical works as a ligand, it induces conformational change in NRLBD and recruits the TIF2-BAP on the plate surface. *p*-Nitrophenyl phosphoric acid (*NPP*) is used as a substrate for BAP. The BAP converts NPP to *p*-nitrophenol (*NP*), which appears *yellow*

2002). They proposed that PMF causes the development of male sex characteristics following an external stimulus such as TBT exposure. However, PMF cannot be the primary factor in the induction of imposex symptoms by TBT. There must be something other factor that directly interacts with TBT in the initial step of imposex induction.

Tributyltin and Triphenyltin as high-affinity ligands for nuclear receptors

Recently, Kanayama et al. (2005), by comprehensive screening of suspected endocrine disruptors versus human nuclear receptors, reported that TBT and TPT are high-affinity ligands for RXR and PPARγ. Their screening method was based on the ligand-dependent interaction between nuclear receptors and coactivators (Kanayama et al. 2003). In the initiation step of transcriptional activation, cognate ligands change the three-dimensional conformations of nuclear receptors (Bourguet et al. 1995; Renaud et al. 1995; Brzozowski et al. 1997; Agostini et al. 1998; Nolte et al. 1998). Next, a coactivator is exclusively recruited to its ligand-bound form of the receptor, but not to the ligand-free form (Fig. 2a). Kanayama et al. developed an in vitro detection method for ligand-dependent interaction between coactivator and nuclear receptors and applied it to the high-throughput screening (Fig. 2b). Using this system, they found that several suspected endocrine disruptors affected multiple nuclear receptors simultaneously. Among them, the effects of organotin compounds on RXR and PPARγ were most obvious. The agonistic effect of TBT on RXR was as strong as that of its endogenous ligand, 9-*cis* retinoic acid, and the effect of TPT on PPARγ was as strong as that of its well-known ligand, rosiglitazone. They also showed that TBT and TPT induced the transactivation function of RXR and PPARγ in mammalian culture cells (Kanayama et al. 2005). The dose range of TBT or TPT that induced transcriptional activation was 10–100 nM; this is almost pharmacologically relevant to the range reported to induce imposex in gastropods.

In mammals, PPARγ binds to DNA as a heterodimer with RXR and plays a central role in adipocyte gene expression and differentiation (Tontonoz et al. 1994). The PPARγ is abundantly expressed in adipocytes, and its ligands induce the efficient conversion of fibroblastic cells to adipocytes, as measured by induction of adipocyte-specific genes and lipid accumulation (Lehmann et al. 1995). In fact, TBT or TPT promotes differentiation of mouse preadipocyte 3T3-L1 cells to adipocytes (Kanayama et al. 2005). Therefore, organotin compounds may have adverse health effects on mammals by disturbing the endocrine processes mediated by the PPARγ/RXR pathway.

However, as I mentioned before, the composition of members of the nuclear receptor superfamily is quite different between vertebrates and invertebrates. The subgroup members of thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR), and PPAR appear to have been late acquisitions during the evolution of the nuclear receptor superfamily (Escriva et al. 1997; Laudet 1997). Therefore, PPARγ might not be present in marine gastropods (Fig. 3). In contrast, RXR is special among the nuclear receptor superfamily. It is widely conserved in the evolutionary tree and its homologue, called ultraspiracle (USP), is found even in arthropods (Laudet 1997).

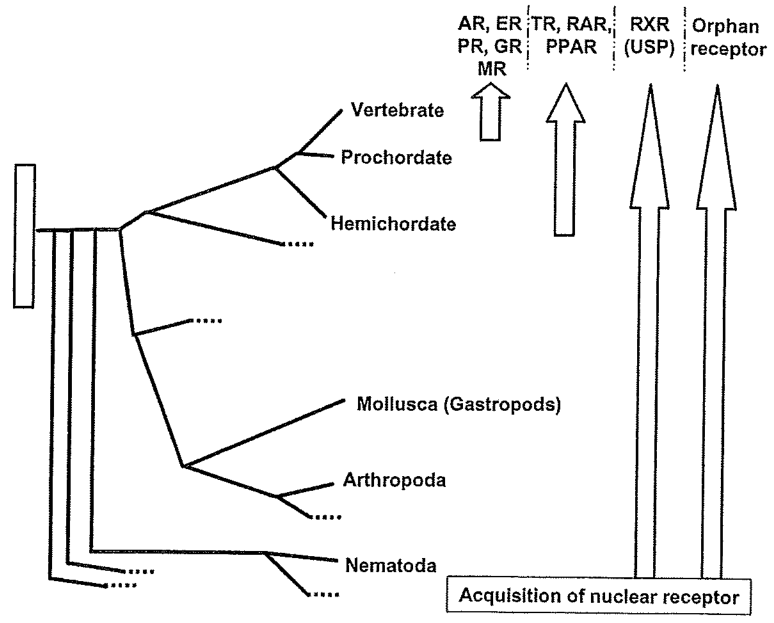

Fig. 3 Evolutionary tree and acquisition of nuclear receptors. Steroid hormone receptors (*AR* androgen receptor, *ER* estrogen receptor, *PR* progesterone receptor, *GR* glucocorticoid receptor, *MR* mineralocorticoid receptor) exist only in vertebrates. The subfamily of TR, RAR, and PPAR are present in vertebrates to hemichordates. In contrast, RXR or its homologue USP exist even in insects and nematodes

Characteristics of gastropod retinoid X receptor

Retinoid X receptor homologue has been cloned from *T. clavigera* (Nishikawa et al. 2004). Gastropod RXR has a DNA binding domain (DBD) composed of two C_2C_2-type zinc finger motifs and a putative LBD in the C-terminal region (Fig. 4a). The highest similarity with other species is in the DBD, where 85–90% of the amino acids residues are identical (Fig. 4b). The LBD of gastropod RXR also shows considerable similarity with that of vertebrate RXRs but has much less similarity with USP, the RXR homologue first found in *D. melanogaster*. Although RXR binds 9-*cis* retinoic acids in organisms ranging from cnidarians (*Tripedalia cystophora*) to vertebrates, USP from arthropods is unable to do so (Heyman et al. 1992; Mangelsdorf et al. 1992; Henrich and Brown 1995; Kostrouch et al. 1998). As expected by the similarity of a gastropod homologue to vertebrate RXR, the binding of gastropod RXR to 9-*cis* retinoic acid has been confirmed experimentally (Nishikawa et al. 2004). The dissociation constant in the binding of 9-*cis* retinoic acid to gastropod RXR is 15.2 nM, which is similar to the values reported for vertebrate RXRs (1–10 nM)

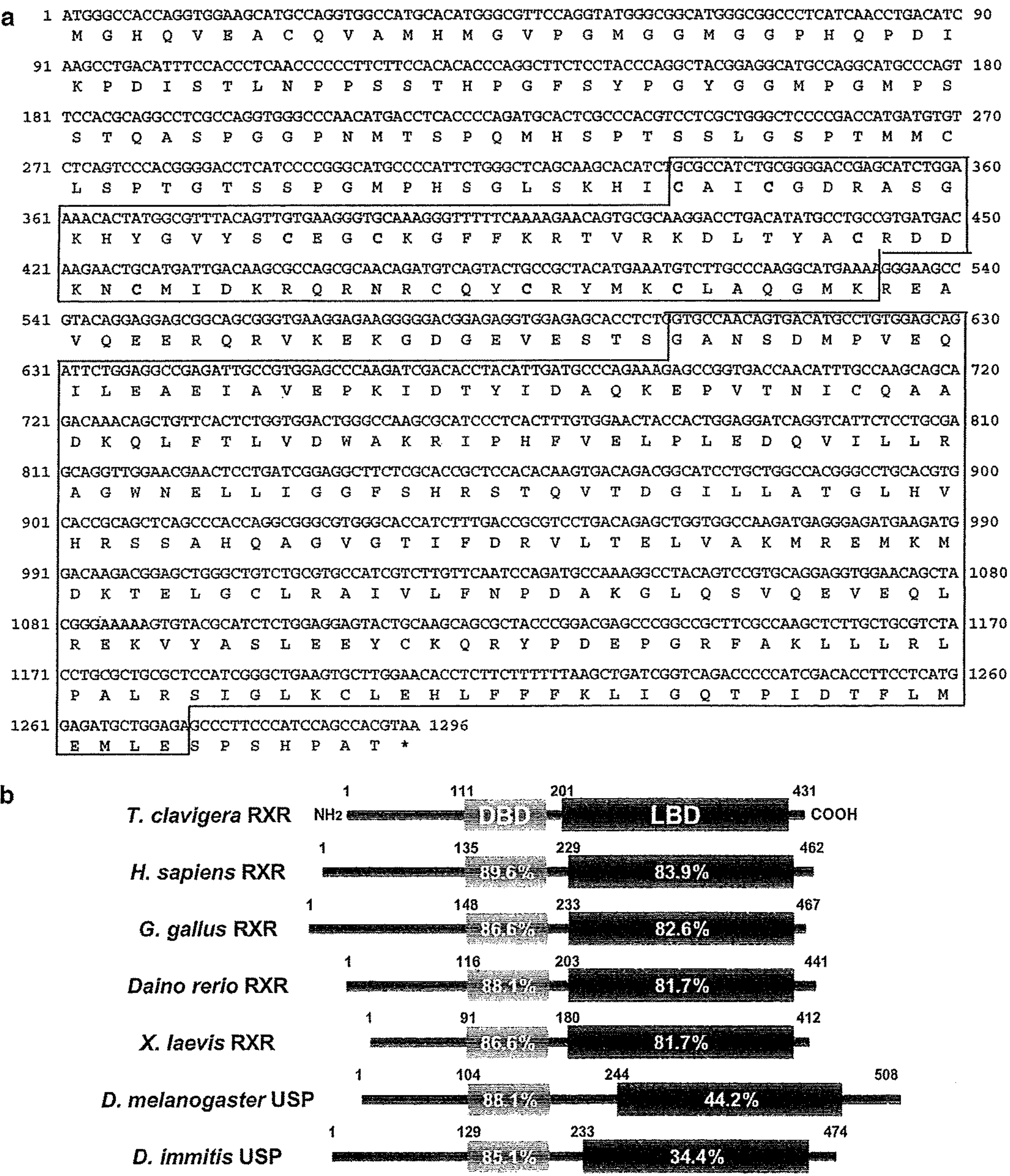

Fig. 4 **a** The entire coding sequence of gastropod RXR. The DNA and LBDs are *boxed*. The database accession number for the sequence is AY704160. **b** A schematic representation of RXR from various species is shown, along with the percentage of identical amino acid residues shared with those of gastropod RXR

Table 1 Inhibitory concentrations of 9-*cis* retinoic acid and organotin compounds in binding of radio-labeled 9-*cis* retinoic acid to human or gastropod RXR

Receptor	Compounds	IC_{50} (μM)
Human RXRα	9-*cis* retinoic acid	0.99
	Tributyltin	0.99
	Triphenyltin	0.85
Gastropod RXR	9-*cis* retinoic acid	0.81
	Tributyltin	8.16
	Triphenyltin	6.49

(Heyman et al. 1992). Gastropod RXR also binds to organotin compounds, even though the 50% inhibitory concentration (IC_{50}) values are larger than for 9-*cis* retinoic acid (Table 1).

Development of imposex in *Thais clavigera* by injection of 9-*cis* retinoic acid

Organotin compounds are potent and efficacious agonistic ligands of the vertebrate nuclear receptors RXR and PPARγ (Kanayama et al. 2005). It is worth noting that receptor activation is observed at nanomolar concentrations, whereas other mechanisms of toxicity (e.g., aromatase inhibition) occur in the micromolar range. Furthermore, there is a functional RXR homologue in gastropods that binds to both 9-*cis* retinoic acid and organotin compounds (Nishikawa et al. 2004). These facts suggest that gastropod imposex is mediated by RXR. Consistent with this prediction, Nishikawa et al. observed that 9-*cis* retinoic acid, like TPT, actually induces imposex in female gastropods (Nishikawa et al. 2004).

So far, certain neuropeptides have been considered as sex hormones in mollusks, as opposed to steroid hormones in vertebrates (LeBlanc et al. 1999). Oberdörster and McClellan-Green have demonstrated that only APGWamide, out of four neuropeptides, induces imposex in the mud snail (Oberdörster and McClellan-Green 2000). They put forward the model that the peptide hormones control the release of fat-soluble hormones, similar to the feedback control of the hypothalamic-pituitary axis in vertebrates (Oberdörster and McClellan-Green 2002). It is possible that APGWamide regulates the expression of some cytochrome P450 (CYP) that catalyzes the transformation of retinoids into active forms such as 9-*cis* retinoic acid.

Perspectives

This review has explored the concept that organotin-induced imposex in marine gastropods is mediated by nuclear receptors. This can be understood in terms of the molecules and mechanisms that regulate male sexual development in mollusks. Vertebrate-type steroid hormone receptors, including AR, are absent in invertebrates, suggesting that androgens may not act as

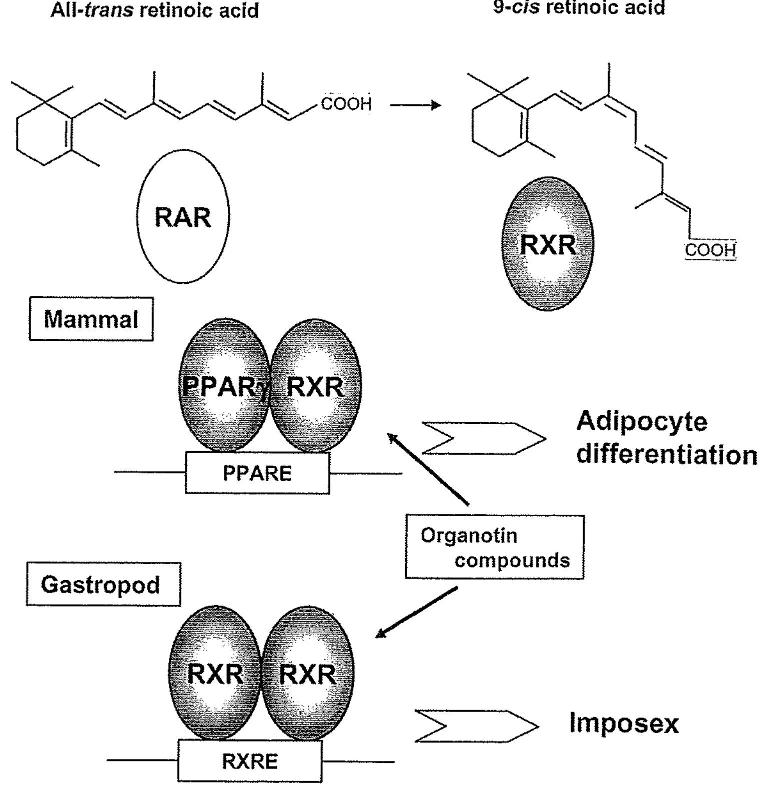

Fig. 5 Effects of organotin compounds in mammals and gastropods via RXR. In mammals, organotin compounds are ligands for both PPARγ and RXR and affect adipocyte differentiation. In gastropods, organotin compounds induce imposex by binding to RXR

male sex hormones. The TBT or TPT stimulates the development of the male genital tract in female gastropods. The TBT and TPT are high-affinity ligands for RXR. Gastropods have a functional homologue of RXR. The 9-*cis* retinoic acid, a natural ligand of RXR, significantly caused the development of imposex in female rock shells. These results suggest that RXR plays an important role in the induction, differentiation, and growth of male genital organs in female gastropods (Fig. 5).

In mammals, RXR is known to act both as a ligand-dependent transcription factor and as a common heterodimer partner for many non-steroid nuclear receptors (Mangelsdorf and Evans 1995). In the cases of some heterodimers, RXR is not activated by its own ligand (Mukherjee et al. 1997). In contrast, synthetic RXR-selective ligands activate RXR homodimer-dependent transcription (Boehm et al. 1995). Because 9-*cis* retinoic acid effectively induces imposex, RXR may function as a homodimer in gastropods. Meanwhile, we do not know whether gastropods inherently possess a pathway for the biosynthesis of retinoic acid. Therefore, we do not know whether 9-*cis* retinoic acid is a real hormone or whether similar derivatives are. We need to identify the active compound responsible for male sexual development in gastropods. Even in mammals, 9-*cis* retinoic acid is difficult to detect in vivo and its action remains obscure (Horton and Maden 1995). The study of retinoids in gastropod imposex may provide some insight into the physiological function of 9-*cis* retinoic acid.

Knock-out mice have provided important information on the physiological functions of these receptors. There are three subtypes of RXR in mammals. RXRα null mice die at embryonic days 12.5–16.5 and exhibit a hypoplastic ventricular myocardium as well as conotruncal and ocular abnormalities (Kastner et al. 1994; Sucov et al. 1994). Approximately 50% of RXRβ null mice die before or at birth, and the remaining male null mutants are sterile, owing to the aberrance of lipid metabolism in the Sertoli cells (Kastner et al. 1996). RXRγ null mice are viable and do not display any abnormalies (Krezel et al. 1996). Dysfunction study using RNAi or homologous recombination in gastropods will be needed to determine the role of RXR in the imposex development.

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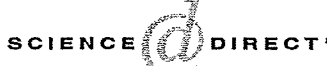

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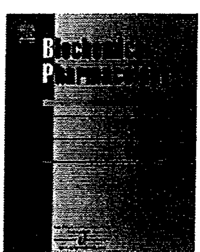

Organotin compounds enhance 17β-hydroxysteroid dehydrogenase type I activity in human choriocarcinoma JAr cells: Potential promotion of 17β-estradiol biosynthesis in human placenta

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TBT, tributyltin
TPT, triphenyltin
17β-HSD I, 17β-hydroxysteroid dehydrogenase type I
17β-HSDs, 17β-hydroxysteroid dehydrogenases
E1, estrone
E2, 17β-estradiol
9cRA, 9-*cis* retinoic acid
atRA, all-*trans* retinoic acid

ABSTRACT

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), are typical environmental contaminants and suspected endocrine-disrupting chemicals because they cause masculinization in female mollusks. However, it remains unclear whether organotin compounds also cause crucial toxicities in human sexual development and reproductive functions. We investigated the effects of 17 tin compounds on the catalytic activity and mRNA expression of 17β-hydroxysteroid dehydrogenase type I (17β-HSD I) in human choriocarcinoma JAr cells. At nontoxic concentrations, both trialkyltins with propyl, butyl or cyclohexyl substituents on the tin atom and triphenyltin (TPT) enhanced 17β-HSD I mRNA transcription and enzyme activity in a dose-dependent fashion. Although tetraalkyltin compounds such as tetrabutyltin and tributylvinyltin also increased the mRNA expression and enzyme activity of 17β-HSD I, the concentrations necessary for activation were >30–100 times greater than those for trialkyltins. Inorganic tin had no effect on the catalytic activity and mRNA expression of 17β-HSD I. Interestingly, diphenyltin and monophenyltin, which are metabolites of TPT, enhanced 17β-HSD I activity with a concomitant increase in mRNA expression, whereas dibutyltin and monobutyltin, which are metabolites of tributyltin, enhanced 17β-HSD I activity without a concomitant increase in mRNA expression. These results suggest that organotin compounds are potent stimulators of 17β-estradiol biosynthesis to enhance 17β-HSD I activity in the human placenta in vitro; the placenta represents a potential target organ for these compounds, whose endocrine-disrupting effects might be the result of local changes in 17β-estradiol concentrations in pregnant women.

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FCS, fetal calf serum
MEM, minimal essential medium
RXR, retinoid X receptor
LG, LG100268
RAR, retinoic acid receptor

1. Introduction

The placenta plays a vital role in maintaining pregnancy by delivering oxygen and nutrients from the maternal circulation to the fetus and by returning fetal metabolites to the mother. In addition, the placenta performs many crucial endocrine functions. For example, the human placenta is the main source of estrogenic steroids during human pregnancy [1].

17β-Hydroxysteroid dehydrogenases (17β-HSDs) catalyze the interconversion of 17-ketosteroids and 17β-hydroxysteroids, such as estrone (E1) to 17β-estradiol (E2), and androstenedione and testosterone. Thus, these members of the short-chain alcohol dehydrogenase protein family catalyze the conversion of low-activity steroids to high activity forms and vice versa. So far, multiple different types of 17β-HSDs have been cloned [2], and these isoenzymes have been found to differ from each other in substrate specificity as well as in tissue distribution and subcellular localization. In the human placenta and ovarian granulosa cells, the type I enzyme (17β-HSD I) is highly expressed [3–5] and catalyzes primarily the reaction from low-activity E1 to the biologically more active form E2. In addition to being found in steroidogenic tissues, 17β-HSD I is present in some estrogen target cells, such as breast [6] and endometrial epithelial cells [7], which suggest its involvement in the regulation of intracellular E2 supplies for estrogen receptors. Given the pivotal functional roles of 17β-HSD I, the developmental and reproductive toxicity of environmental contaminants known to have endocrine-disrupting effects plausibly might involve 17β-HSD I.

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been widely used as biocides, agriculture fungicides, wood preservatives, and disinfecting agents in circulating industrial cooling waters, as well as antifouling paints for marine vessels [8,9]. There are many reports of the biological effects of organotin compounds, which vary in their toxic effects on eukaryotes. One of the most notable toxicities in sexual development and reproduction is that of TBT- and TPT-mediated endocrine disruption in some species of gastropods [10,11]. This phenomenon is known as "imposex"—the superimposition of male genitalia on female animals. Therefore, these organotin compounds are suspected to cause endocrine-disrupting effects in mammals, including humans. Human exposure to organotin compounds may result from consumption of organotin-contaminated meat and fish products or occupational exposure during the manufacture and formulation of organotin compounds or the application and removal of organotin-containing paints [12,13]. The possible exposure of humans to organotins therefore has prompted great concern about potential toxicities.

To facilitate the application of current knowledge of the toxicity of organotin compounds to sexual development and reproduction in humans, we assessed the possible effects of 17 tin compounds on E2 production and mRNA expression of 17β-HSD I in human placental cells by using human choriocarcinoma JAr cells. We discuss the potential toxicity of organotin compounds as endocrine disruptors in humans.

2. Materials and methods

2.1. *Chemicals and cell culture*

Tin compounds tested in this study are listed in Table 1. 9-*cis* retinoic acid (9cRA) and all-*trans* retinoic acid (atRA) were from Wako Pure Chemicals (Osaka, Japan). LG100268 (LG, >95% pure) was obtained from Astellas Pharma (Tokyo, Japan). All chemicals were dissolved in DMSO (Wako Pure Chemicals). The human choriocarcinoma cell line JAr was obtained from American Type Culture Collection (ATCC; Rockville, MD). JAr cells (ATCC No. HTB-144) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM pyruvate, 4.5 g/l glucose, and 10% fetal calf serum (FCS). JEG-3 cells (ATCC No. HTB-36) were cultured in minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid solution (Invitrogen, Carlsbad, CA), and 10% FCS. To determine the effect of tin compounds on [^{3}H]thymidine incorporation and mRNA expression of JAr cells, cells were seeded, precultured for 24 h, and then treated with either various concentrations of tin compounds in 0.1% DMSO or vehicle alone (0.1% DMSO) for another 24 or 48 h. In control experiments, 0.1% DMSO did not affect the [^{3}H]thymidine incorporation, catalytic activity, and mRNA expression of 17β-HSD I.

2.2. *[^{3}H]thymidine uptake assay*

To determine the cytotoxicity of tin compounds, JAr cells (10^3 cells/well) were seeded in 96-well plates. After 24 h, cells were treated with various concentrations of these compounds for another 48 h. Each culture was pulsed with 20 kBq of [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) for 2 h before harvesting, and the radioactivity incorporated into cells was determined by liquid scintillation. A nontoxic concentration of a tin compound was defined as a concentration at which the uptake of [^{3}H]thymidine was ≥80% that seen with the vehicle alone.

2.3. *17β-HSD I activity measurements*

JAr cells (3×10^4 cells/well) were plated in 24-well plates. After 24 h of culture, JAr cells were treated with various concentrations of tin compounds for a further 48 h. At the end point of

Table 1 – Tin compounds tested in this study

Tin compounds	Abbreviation	Structure	Purify (%)	CAS No.	Maximum nontoxic concentration[a]	Source
Trimethyltin chloride	TMTCl	CH_3, H_3C–Sn–Cl, CH_3	>98	1066-45-1	1 μM	Aldrich Chemicals
Triethyltin bromide	TETBr	Sn, Br	>97	2767-54-6	100 nM	Aldrich Chemicals
Tripropyltin chloride	TPrTCl	Sn, Cl	>98	2279-76-7	30 nM	Merck
Tributyltin chloride	TBTCl	Sn, Cl	>95	1416-22-0	100 nM	Tokyo Kasei Kogyo
Triphenyltin chloride	TPTCl	Sn, Cl	>95	639-58-7	100 nM	Aldrich Chemicals
Tricyclohexyltin hydroxide	TChTOH	Sn, HO	>99	13121-70-5	30 nM	Aldrich Chemicals
Trioctyltin hydride	TOTH	Sn, H	>95	869-59-0	>10 μM	Tokyo Kasei Kogyo
Butyltin trichloride	$MBTCl_3$	Cl, Cl–Sn, Cl	>95	1118-46-3	>10 μM	Aldrich Chemicals
Dibutyltin dichloride	$DBTCl_2$	Cl–Sn, Cl	>97	683-18-1	30 nM	Tokyo Kasei Kogyo
Tetrabutyltin	TeBT	Sn	>93	1461-25-2	3 μM	Aldrich Chemicals
Phenyltin trichloride	$MPTCl_3$	Cl, Cl, Sn, Cl	>98	1124-19-2	3 μM	Aldrich Chemicals
Diphenyltin dichloride	$DPTCl_2$	Cl, Sn, Cl	>96	1135-99-5	300 nM	Aldrich Chemicals
Tributyltin hydride	TBTH	Sn, H	>98	688-73-3	100 nM	Aldrich Chemicals
Tributyltin bromide	TBTBr	Sn, Br	>90	1461-23-0	100 nM	Aldrich Chemicals
Triphenyltin hydroxide	TPTOH	Sn, HO	>95	76-87-9	100 nM	Aldrich Chemicals
Tributylvinyltin	TBVT	Sn	>97	7486-35-3	>10 μM	Tokyo Kasei Kogyo
Tin chloride	$SnCl_4$	Cl, Cl–Sn–Cl, Cl	>98	7646-78-8	>10 μM	Wako Pure Chemicals

[a] Maximum nontoxic concentration of each tin compound was defined as the maximum concentration at which the uptake of $[^3H]$thymidine was ≥80% of that of the vehicle alone.

each treatment, cells were rinsed with fresh serum-free culture medium and then added 0.5 ml of fresh serum-free culture medium supplemented with 1 μM E1 (Sigma, St. Louis, MO). After incubation for 4 h at 37 °C in an atmosphere of 5% CO_2, culture media were collected, and the total E2 content was determined by assay with a Correlate-EIA 17β-estradiol Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI).

2.4. *Quantitative reverse transcription-PCR (RT-PCR)*

JAr cells were treated with various tin compounds in regular culture medium supplemented with 5% charcoal-stripped FCS instead of 10% normal FCS, and then total RNA was extracted from the cells by using TRIzol reagent (Invitrogen). mRNA expression of 17β-HSD I in JAr cells was determined by quantitative RT-PCR. We reverse-transcribed 5 μg total RNA extracted from JAr cells in a total volume of 20 μl by using SuperScript III reagent (Invitrogen) and oligo-(dT) as primer and incubating for 1 h at 42 °C. After termination of cDNA synthesis, each reaction mixture was diluted with the addition of 80 μl TE buffer. Aliquots (2 μl) of diluted reverse-transcription products were amplified in a reaction mixture containing QuantiTect SYBR Green PCR reagent (Qiagen, Valencia, CA) and 0.5 μM of each primer using LightCycler (Roche Diagnostics, Mannheim, Germany). After preincubation of reaction mixtures at 95 °C for 15 min, PCR amplification was performed with 35–40 cycles of denaturation at 95 °C for 15 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 10 s. Primers used were for human 17β-HSD I (HSD17B1), 5′-GGGCTGCCTTTCAATGACGTTT-3′ and 5′-ATCAGGCTCAAGTGGACCCCAA-3′, and human β-actin, 5′-CTACGAGCTGCCTGACGGC-3′ and 5′-GCCACAGGACTCCATGCCC-3′.

2.5. *Statistics*

Data were analyzed with Tukey's multiple comparisons test by using SPSS software (Chicago, IL). Control and treatment group data always were obtained from equal numbers of replicate experiments, and experiments were performed independently at least twice. Values at which P was <0.05 were considered statistically significant.

3. Results

3.1. *Effects of organotin compounds on DNA synthesis in JAr cells*

To confirm the nontoxic concentration ranges of 17 tin compounds (Table 1) and to determine whether treatment with organotin compounds was associated with cytotoxic effects, we performed DNA synthesis assays. JAr cells were treated for 48 h with tin compounds at various concentrations, and DNA synthesis was evaluated by [^{3}H]thymidine incorporation. To help interpret the results, we classified these experiments into three groups as follows: Group I, comparison of different structures of alkyl and aryl chains in trialkylated and triarylated tin compounds; Group II, comparison of different numbers of alkyl or aryl chains in butyltin and phenyltin compounds; and Group III, comparison of different fourth functional groups on the tin of TBT and TPT (Fig. 1).

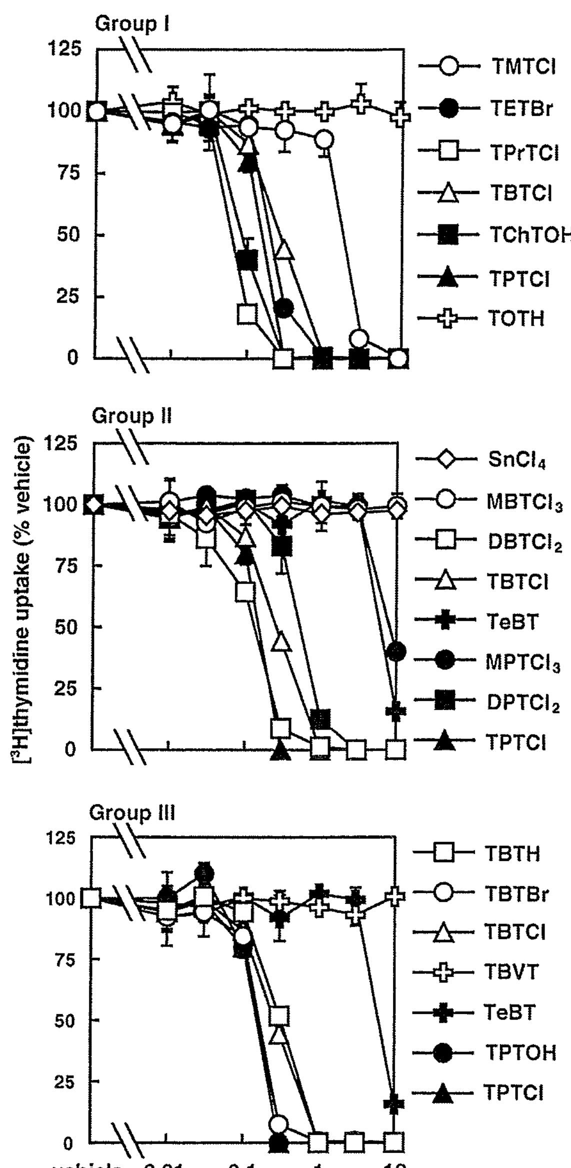

Fig. 1 – Effect of organotin compounds on DNA synthesis in JAr cells. Cells were treated with various concentrations of tin compounds for 48 h. Results are expressed as mean ± 1 S.D. of triplicate cultures. The radioactivity in vehicle-only cells, calculated from all experiments, was 111,709 ± 6182 cpm (*n* = 15). Group I, comparison of different structures of alkyl and aryl chains in trialkylated and triarylated tin compounds; Group II, comparison of different numbers of alkyl or aryl chains in butyltin and phenyltin compounds; and Group III, comparison of different fourth functional groups on the tin of TBT and TPT. The abbreviation for each compound used are indicated in Table 1.

Many of the Group I trialkylated and triarylated tin compounds, which have ethyl, propyl, butyl, cyclohexyl, or phenyl groups on the tin atom, were highly toxic, and

exposure to >100–300 nM significantly inhibited [^{3}H]thymidine incorporation in JAr cells. Although TMTCl was one of the less toxic chemicals of Group I, a striking reduction of [^{3}H]thymidine incorporation to 5% of the control value occurred after treatment with >1 μM. TOTH had no significant effect on [^{3}H]thymidine incorporation at a concentration range of 10 nM to 10 μM. Among the Group II chemicals, the cytotoxicity of $DBTCl_2$ was nearly as high as that of the most highly toxic trialkyltins. $DPTCl_2$ was also toxic but less so than $DBTCl_2$. Although TeBT and $MPTCl_3$ were less toxic than other Group II compounds, they induced marked reduction of [^{3}H]thymidine incorporation at 10 μM. $SnCl_4$ and $MBTCl_3$ showed no effect, even at concentrations of 10 μM. Among the Group III chemicals, the TBT and TPT derivatives were similar in toxicity, and exposure to doses of 300 nM decreased [^{3}H]thymidine incorporation to <50% of control levels. TBVT showed no significant effect at the concentration range of 10 nM to 10 μM. Using these results as a guide, we established the maximal nontoxic concentration of each compound for use in investigating possible effects on the 17β-HSD I activity of JAr cells (Table 1).

3.2. *Effect of organotin compounds on 17β-HSD I activity in JAr cells*

At lethal concentrations, at which uptake of [^{3}H]thymidine was <10% of control levels, all organotin compounds abolished E2 production because of extinction of the cells. All tested TBT and TPT derivatives (Group III) were active and induced 17β-HSD I activity (Fig. 2): exposure to 100 nM of each of these organotin compounds caused statistically significant increases in 17β-HSD I activity in JAr cells. There were no significant differences in 17β-HSD I activity among the TBT and TPT derivatives (Group III), suggesting that the ligand on the trialkylated and triarylated tin compounds (as long as it is not an alkyl or aryl group) is relatively unimportant to stimulation of endocrine functions. However, the presence of a fourth alkyl group on the tin atom decreased the potency of the organotin compounds in inducing 17β-HSD I activity, because both TeBT and TBVT failed to stimulate this placental function at doses of <100 nM (Fig. 2, Group III). Among the other trialkyltin compounds (Group I), TETBr, TPrTCl, and TChTOH were significantly active. Metabolites of both TBTCl and TPTCl ($MBTCl_3$, $DBTCl_2$, $MPTCl_3$ and $DPTCl_2$; Group II) also altered 17β-HSD I activity, but the level of activation decreased in proportion to the dealkylation or dearylation of these organotin compounds (mono- < di- < tri-). These results suggest that the potency of the effects induced by organotin compounds is related to both the number and structure of the alkyl and aryl groups.

3.3. *Effect of organotin compounds on mRNA expression of 17β-HSD I (HSD17B1) in JAr cells*

We investigated the tin compound-induced mRNA expression of 17β-HSD I (*HSD17B1* gene) in JAr cells at either the concentration that elicited the greatest response in catalytic activity or the maximal nontoxic concentration. Except for

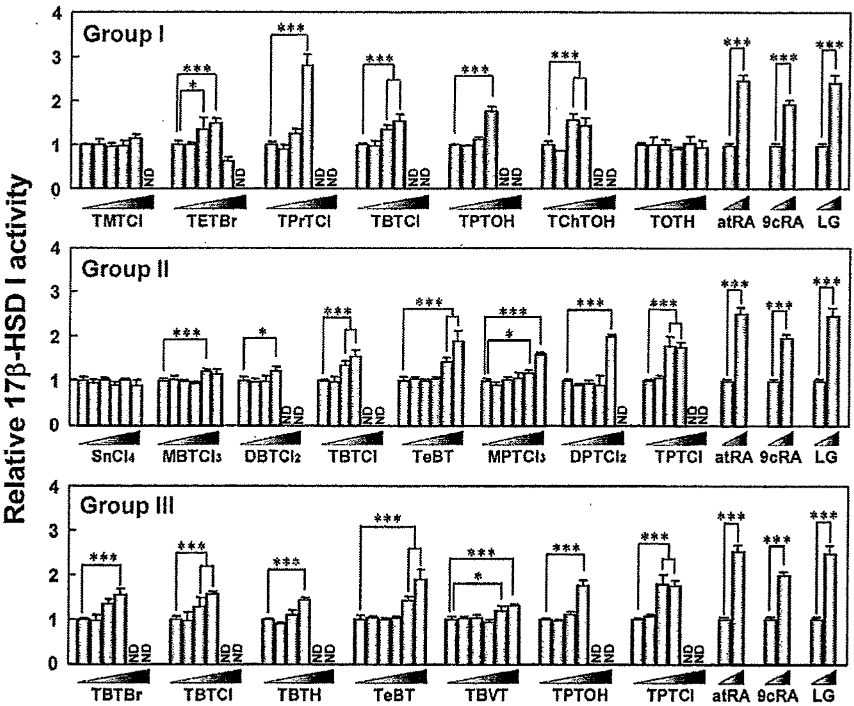

Fig. 2 – Effects of tin compounds on 17β-HSD I activity in JAr cells. Cells were treated for 48 h with tin compounds at various concentrations (0 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) or with 0 or 1 μM of atRA, 9cRA, or LG. Results are expressed as mean ± 1 S.D. of triplicate cultures. The 17β-HSD I activity (amount of converted E2) in vehicle-only cells, calculated from all experiments, was 4.44 ± 1.01 ng/well/4 h (*n* = 18). Groups I, II, and III correspond to the groups described in the legend for Fig. 1. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.005$ indicate values significantly different from vehicle-control values. ND, not detectable.

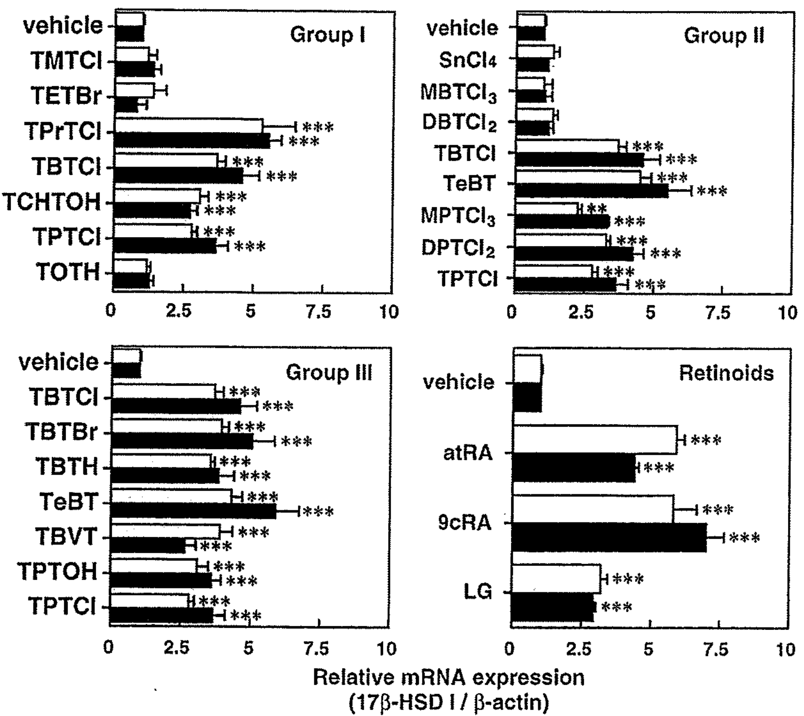

Fig. 3 – Effects of tin compounds on the mRNA expression of 17β-HSD I (HSD17B1) in JAr cells. Total RNA was isolated from JAr cells treated with tin compounds for 24 h (open bars) and 48 h (solid bars). The doses of each compound were: 100 nM of TETBr, TPrTCl, TBTCl, TChTOH, TPTOH, TPTCl, TBTH, TBTBr and $DBTCl_2$; 1 μM of TMTCl, $MBTCl_3$, $DPTCl_2$, 9cRA, atRA and LG; and 10 μM of TOTH, $SnCl_4$, $MPTCl_3$, TeBT and TBVT. The relative mRNA levels for each condition were determined by quantitative RT-PCR assays for each of the 3 independent cultures (see Section 2). Results are expressed as means ± 1 S.D. of three independent cultures. Groups I, II, and III correspond to the groups described in the legend for Fig. 1. $^{**}P < 0.01$; and $^{***}P < 0.005$ represents values significantly different from vehicle-control values.

TETBr, $MBTCl_3$, and $DBTCl_2$, the organotin compounds that significantly enhanced the catalytic activity of 17β-HSD I also significantly increased its mRNA expression. However, the mRNA effects were much more pronounced than the changes in catalytic activity (Figs. 2 and 3). Furthermore, atRA and 9cRA, which are known to enhance 17β-HSD I activity with mRNA expression, also showed their induction of mRNA expression more obviously than that of catalytic activity (Figs. 2 and 3).

In a recent study, we demonstrated that some organotin compounds function as agonists for retinoid X receptor (RXR), a nuclear receptor for retinoids, to stimulate the mRNA expression of human placental aromatase and human chorionic gonadotropin in human choriocarcinoma cells [14]. As shown in Figs. 2 and 3, organotin compounds that induced the transactivation function of RXR in our previous study (TBT and TPT derivatives, TPrTCl, TChTOH, TeBT, and TBVT) also enhanced 17β-HSD I mRNA transcription and enzyme activity. In addition, a synthetic RXR-specific ligand LG also enhanced 17β-HSD I enzyme activity and mRNA transcription (Figs. 2 and 3). These results suggest that these organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

However, organotin compounds that did not induce the transactivation function of RXR in our previous study (TETBr, $MBTCl_3$, $DBTCl_2$, $MPTCl_3$ and $DPTCl_2$) also significantly enhanced 17β-HSD I enzyme activity. Interestingly, $MPTCl_3$ and $DPTCl_2$ significantly enhanced mRNA expression of 17β-HSD I, whereas TETBr, $MBTCl_3$, and $DBTCl_2$ had little effect on mRNA expression (Fig. 3). These results indicate that the observed organotin-induced alterations in 17β-HSD I activity are due not only to regulation at the mRNA level but also another mechanism.

4. Discussion

Recently, organotin compounds have become recognized as endocrine-disrupting chemicals, because numerous marine organisms have been shown to exhibit sexual abnormalities after exposure to TBT or TPT. In gastropod mollusks, which are among the species most sensitive to organotin compounds, these chemicals have been demonstrated to induce the superimposition of male sex organs, such as a penis and/or a vas deferens, over female sex organs, a phenomenon known as imposex [10,11]. It has been theorized that TBT increases androgen levels through inhibition of aromatase activity or suppression of androgen excretion [15–17]; nevertheless this theory is not well founded. Although these organotin compounds also are reported to inhibit the catalytic activity of human steroidgenic enzymes, including aromatase [18,19], 3β-hydroxysteroid dehydrogenase type II, 5α-reductase type II, and 17β-HSD I and III [20,21], the concentrations effective for the inhibition of these enzymes were relatively high (>1 μM)

and generally toxic to mammalian cells [22–24], including human choriocarcinoma cells (Fig. 1). Therefore, in regard to effects on humans, we have to distinguish between the nonspecific cellular toxicity of organotins and their inhibition of steroidgenic enzymes. We previously demonstrated that nanomolar concentrations (i.e., 3–100 nM) of some organotin compounds, including TBT and TPT, enhance aromatase activity, which catalyzes the conversion androgen to estrogen, and that this increase in enzymatic activity occurs concurrently with increases in mRNA expression and E2 biosynthesis from androstenedione in human choriocarcinoma cells [14,22]. In addition, our current study showed that many of the same organotin compounds also enhance 17β-HSD I activity, which predominantly catalyzes the conversion of the weakly estrogenic chemical E1 into the strongly estrogenic compound E2 in JAr cells (Figs. 2 and 3). Our findings suggest that the endocrine-disrupting action of these organotin compounds in pregnant women may be to promote the biosynthesis of E2 in the placenta, an effect opposite to that theorized in gastropods.

Our current study has demonstrated that organotin compounds alter E2 biosynthesis in human placental cells in vitro. Although several reports have established the in vivo reproductive toxicity of organotin compounds in rodents [25–28], there are no reports on whether organotin-induced production of placental E2 is associated with teratogenetic effects. Accordingly, it remains unclear which endocrine-disrupting effects or malformations result, at least in part, from organtoin-induced local changes in E2 concentrations of the placenta in vivo. Further, the in vivo endocrine effects of environmental contaminants on the human placenta are difficult to estimate from animal studies, particularly those involving rodents, because the endocrine functions of the placenta vary considerably among different species. In particular, estrogen biosynthesis during pregnancy in humans is much different from that in rodents. In humans, ovarian function gradually declines after fertilization, as the placenta becomes the primary site of estrogen biosynthesis during pregnancy [29]. In contrast to the process in humans, the ovary (not the placenta) is the main source of estrogen during pregnancy in rodents, because the placenta of rodents expresses neither aromatase nor 17β-HSD I [30–32]. It has been suggested that rodents are therefore unsuitable for evaluating the effects of environmental contaminants on estrogen biosynthesis in the human placenta. The regulation of estrogen biosynthesis in placenta is very important for human embryo because altering placental function can cause permanent effects in the embryo. For example, the lack of placental aromatase causes female pseudohermaphroditism, as is seen in patients with aromatase deficiency [33]. Consequently, there is an urgent need to establish effective tools to evaluate the endocrine-disrupting effects and teratogenicity of environmental contaminants that induce changes in local estrogen concentrations of the placenta in vivo.

Several stimuli, such as cAMP analogues [34], the natural retinoic acid receptor (RAR) ligand atRA [35], and the natural RAR and RXR ligand 9cRA [36], induce the mRNA expression of 17β-HSD I, thereby increasing its activity. Organotin compounds, such as TBT and TPT, have no effect on intracellular cAMP production [22]. In a recent study, we demonstrated that some organotin compounds function as RXR agonists to stimulate the mRNA expression of human placental aromatase and human chorionic gonadotropin in human choriocarcinoma cells [14]. These chemicals bind directly to the ligand-binding domain of RXR and function as transcriptional activators. The RXR-agonistic organotin compounds also enhanced the expression of 17β-HSD I mRNA in the present study. The level of activation was nearly equal to the level of RXR activation induced by these compounds [14]. The RAR response element is located at −512 to −479 bp in the promoter region of 17β-HSD I [35], but the RXR response element has not yet been identified. However, Ro41, a specific RAR antagonist, fails to abrogate 9cRA-induced expression of 17β-HSD I mRNA [36]. In addition, we found that the RXR-specific ligand LG consistently enhanced 17β-HSD I enzyme activity and mRNA transcription (Figs. 2 and 3). Unlike 9cRA, these organotin compounds are RXR-specific and do not activate the RAR pathway [14]. In light of these findings, although transcriptional regulation in the 17β-HSD I promoter is not yet fully understood, we suggest that the cis-elements which have a response to the RXR-dependent signaling pathway may be located in the promoter region and that RXR-agonistic organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

We assayed 17 tin compounds for their ability to induce both the catalytic activity and mRNA expression of 17β-HSD I in JAr cells. The concentrations needed to induce these two features of 17β-HSD I did not differ significantly among the TBT and TPT derivatives. Compared with those for the TBT and TPT derivatives, approximately 30- to 100-fold higher concentrations of tetraalkyltin compounds (e.g., TeBT and TBVT) were needed to affect 17β-HSD I activity or transcript levels. This observation may indicate that tetraalkyltin compounds are metabolically converted to trialkyltins, which are the active form, in the cells. This hypothesis is supported, in a broad sense, by previous results that show that organotin compounds undergo dealkylation by the microsomal monooxygenase system, which is dependent on cytochrome P450 in the liver and other organs [37–39]. In addition, our previous study suggested that it may be necessary for activation of RXR by these tetraalkyltin compounds to metabolically convert them into the active dealkylated form (e.g., TBT) in cells, because the presence of a fourth alkyl group on the tin atom interferes with the binding of alkyltin compounds to the receptor [14]. These findings support our speculation that these organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

Tin compounds that failed to act as RXR agonists ($MBTCl_3$, $DBTCl_2$, $MPTCl_3$, $DPTCl_2$ and TETBr) [14] also significantly increased the catalytic activity of 17β-HSD I. Strangely enough, metabolites of TPT ($MPTCl_3$ and $DPTCl_2$) significantly enhanced mRNA expression of 17β-HSD I, whereas metabolites of TBT ($MBTCl_3$ and $DBTCl_2$) and TETBr had little effect on mRNA expression (Figs. 2 and 3). It remains unclear why these organotin compounds enhanced the activity of 17β-HSD I with or without altering its mRNA expression. At least, the induction appears due to a mechanism other than activation of RXRs.

To our knowledge, our study is the first to show that organotin compounds potentially promote estrogenic action to enhance 17β-HSD I activity in human placenta. However, the mRNA changes that the compounds induced were not comparable to the changes in catalytic activity. Consequently, we conclude that the observed organotin-induced alterations in JAr cells are due to other mechanisms in addition to regulation of 17β-HSD I mRNA levels. The toxic mechanisms of organotin compounds appear very intricate. For instance, organotin compounds function as inhibitors of steroidogenic enzymes [18–21] and RXR ligands [14] but also have been shown to enhance histone acetyltransferase activity [40]. Future studies need to clarify the precise mechanism of action of organotin compounds in human endocrine disruption in vitro and in vivo.

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Organotin Compounds Promote Adipocyte Differentiation as Agonists of the Peroxisome Proliferator-Activated Receptor γ/Retinoid X Receptor Pathway

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ABSTRACT

Nuclear receptors play important roles in the maintenance of the endocrine system, regulation of organ differentiation, and fetal development. Endocrine disruptors exert their adverse effects by disrupting the endocrine system via various mechanisms. To assess the effects of endocrine disruptors on nuclear receptors, we developed a high-throughput method for identifying activators of nuclear receptors. Using this system, we found that triphenyltin and tributyltin were activators of peroxisome proliferator-activated receptor (PPAR) γ and retinoid X receptor. Because PPARγ is a master regulator of adipocyte differentiation, we assessed the effect of organotin compounds on preadipocyte 3T3-L1 cells. We found that organotin compounds stimulated differentiation of 3T3-L1 cells as well as expression of adipocyte marker genes.

An endocrine disruptor is an exogenous substance or mixture that alters functions of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or (sub)populations (WHO, 1996). Many naturally occurring and synthetic compounds, including DDT and its metabolites, polychlorinated biphenyls, and some alkylphenols, have hormonal activities (Sohoni and Sumpter, 1998; Nishihara et al., 2000; Gray et al., 2001; Sanderson et al., 2002). Although the levels of natural hormones are precisely regulated metabolically, synthetic chemicals elude this regulation to stimulate organs by mechanisms different from those of natural hormones.

The importance of nuclear receptors in endocrine function has been well established by many studies. The human genome contains at least 48 members of the nuclear receptor family (Chawla et al., 2001), and various chemicals bind to nuclear receptors and influence the expression of target genes (Blair et al., 2000; Sultan et al., 2001). To evaluate the effects of numerous synthetic chemicals on many nuclear receptors, we developed the CoA-BAP system, a high-throughput method for identifying nuclear receptor ligands (Kanayama et al., 2003). In the present study, we applied the CoA-BAP system to the evaluation of 16 human nuclear receptors and 40 suspected endocrine disruptors. We found that organotin compounds such as triphenyltin (TPT) and tributyltin (TBT) strongly activated retinoid X receptor (RXR) and PPARγ.

Organotin compounds have been used as agricultural fungicides, rodent repellents, and molluscicides and in antifouling paints for ships and fishing nets (Piver, 1973; Fent, 1996). These widespread uses have resulted in the release of increasing amounts of organotins into the environment. Although the toxicity of organotins has been reviewed extensively (Boyer, 1989), the molecular target of organotins has not yet been identified.

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ABBREVIATIONS: BAP, bacterial alkaline phosphatase; TPT, triphenyltin; TBT, tributyltin; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; LXR, liver X receptor; RT-PCR, reverse transcription-polymerase chain reaction; FXR, farnesoid X receptor; ERR, estrogen-related receptor; ER, estrogen receptor; TR, thyroid hormone receptor; RAR, retinoic acid receptor; VDR, vitamin D receptor; TIF2, transcriptional intermediary factor 2; hRXR, human retinoic acid receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; Dex, dexamethasone; Rosi, rosiglitazone; LG100268, 6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8- tetrahydronaphthalen-2-yl)cyclopropyl)pyridine-3-carboxylic acid; TO-901317, *N*-(2,2,2-Trifluoroethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethylethyl)phenyl]benzenesulfonamide; GW501516, 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl) phenoxy-acetic acid.

Here, we show that TPT and TBT are high-affinity ligands for RXR and PPARγ. Organotin compounds act as agonists of both RXRα and PPARγ in mammalian reporter gene assays and induce the expression of PPARγ target genes. PPARγ forms a heterodimer with RXR and binds to a defined DNA sequence in the promoter region of target genes (Mangelsdorf and Evans, 1995). PPARγ is activated by a variety of fatty acids and a class of synthetic antidiabetic agents, the thiazolidinediones (Lehmann et al., 1995). PPARγ serves as an essential regulator for adipocyte differentiation and lipid storage in mature adipocytes (Tontonoz et al., 1994). In light of these previous findings, we evaluated the effects of TPT and TBT on adipogenesis and found that organotins stimulate the differentiation of preadipocyte 3T3-L1 cells to adipocytes. Our data suggest that organotins exert their toxic effects through activation of the PPARγ/RXR signaling pathway.

Materials and Methods

Plasmids. The ligand-binding domains (LBDs) of the human nuclear receptors PPARα (codons 168–468; GenBank accession no. L02932), PPARγ1 (177–477; L40904), PPARδ (139–441; L07592), liver X receptor (LXR)α (167–447; U22662), and LXRβ (155–461; U07132) were amplified by RT-PCR from human liver mRNA as the template; the LBDs of human farnesoid X receptor (FXR) (193–472; U68233) and human estrogen-related receptor (ERR)γ (194–458; AF094518) were amplified similarly from human kidney mRNA and that of human ERRβ (195–434; AF094517) was amplified from human testis mRNA. The DNA sequences of the amplified fragments were confirmed by sequencing after subcloning into pGEX-4T (Amersham Biosciences Inc., Piscataway, NJ). The expression vectors for the human nuclear receptors estrogen receptor (ER)α/β, thyroid hormone receptor (TR)α, retinoic acid receptor (RAR)α/γ, RXRα/γ, vitamin D receptor (VDR), and human TIF2 were described previously (Kanayama et al., 2003). For expression in mammalian culture cells, the LBD of hRXRα was fused to the C-terminal end of the GAL4 DNA binding domain (amino acids 1–97) in the pBK-CMV expression vector (Stratagene, La Jolla, CA). The expression plasmid of (GAL4-DBD)-PPARγ (pM-mPPARγ1) and the luciferase reporter plasmid p4xUAS-tk-luc (Kamei et al., 2003) were kind gifts from Dr. Y. Kamei (National Institute of Health and Nutrition, Tokyo, Japan).

Chemical Reagents. Diethyl phthalate, triphenyltin chloride, nitrofen, 4-nonylphenol, octachlorostyrene, permethrin, triphenylmethane, and triphenylethylene were purchased from Kanto Chemical (Tokyo, Japan). Amitrole, 2,4-dichlorophenoxy acetic acid, 1,2-dibromo-3-chloropropane, γ-hexachlorocyclohexane (lindane), pentachlorophenol, dihexyl phthalate, di-*n*-pentyl phthalate, dipropyl phthalate, 2,4-dichlorophenol, 4-nitrotoluene, and bisphenol A were purchased from Tokyo Kasei (Tokyo, Japan). Chenodeoxycholic acid, 1α,25-dihydroxy cholecalciferol, lithocholic acid, all-*trans* retinoic acid, 9-*cis* retinoic acid, and 3,3′,5-triiodo-L-thyronine were purchased from Sigma-Aldrich (St. Louis, MO). 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2, rosiglitazone, and TO-901317 were purchased from Cayman Chemical (Ann Arbor, MI). GW501516 was purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan). The 40 chemicals tested and the abbreviations used for them are listed in Table 1.

Preparation of Proteins. The histidine-tagged fusion protein human TIF2 NID-BAP, in which the nuclear receptor interaction domain of TIF2, was ligated to the bacterial alkaline phosphatase (BAP), was expressed in *Escherichia coli* BL21 (DE3) cells and purified on Ni-nitrilotriacetic acid agarose resin (QIAGEN, Valencia, CA). Except for LXRα/β and FXR, the glutathione *S*-transferase fusion proteins were expressed in the *E. coli* BL21 (DE3) pLysS cells; LXRα/β and FXR were expressed in *E. coli* JM109 pRIL cells. The glutathione *S*-transferase fusion proteins were purified by using glutathione-Sepharose 4B (Amersham Biosciences Inc.).

CoA-BAP System. Detection of ligand-dependent interaction between nuclear receptors and TIF2 was carried out as described previously (Kanayama et al., 2003) but with slight modification. In brief, 2 μg of nuclear receptor protein diluted in 100 μl of carbonate buffer (100 mM $NaHCO_3$, pH 8.4) was incubated in the well of a 96-well polystyrene microtiter plate (MaxiSorp; Nalge Nunc International, Rochester, NY) at 4°C overnight. The plate was washed three times with 120 μl of buffer A (20 mM Tris-HCl, 100 mM KCl, 0.25 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol, and 0.05% Tween 20, pH 7.4), and then 100 μl of TIF2-BAP fusion protein (30 μg/ml) in buffer A was added to a well with the test chemical. After 1-h incubation at 4°C, the plate was washed three times with 120 μl of buffer B (50 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, and 0.10% Nonidet P-40, pH 7.2). The enzyme reaction was started by the addition of 100 μl of substrate solution (10 mM *p*-nitrophenyl phosphate in 100 mM Tris-HCl, pH 8.0). After incubation at 37°C for 30 to 90 min, the reaction was stopped by addition of 25 μl of 0.5 N NaOH. Finally, the absorbance at 405 nm was measured with a plate reader (MultiskanJX; Thermo Labsystems, Helsinki, Finland).

Cell Culture. Mouse 3T3-L1 (Dainippon Pharmaceutical, Osaka, Japan) and mouse NIH-3T3 (clone 5611, JCRB0615; Japanese Cancer Research Resources Bank, Osaka, Japan) fibroblasts were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% calf serum

TABLE 1
Suspected endocrine disruptors tested in this study

No.	Compound	Abbreviation	CAS No.
1	Diethyl phthalate	DEP	84-66-2
2	Dipropyl phthalate	DPrP	131-16-8
3	Di-*n*-butyl phthalate	DBP	84-74-2
4	Di-*n*-pentyl phthalate	DPP	131-18-0
5	Dihexyl phthalate	DHP	84-75-3
6	Diethylhexyl phthalate	DEHP	117-81-7
7	Dicyclohexyl phthalate	DCHP	84-61-7
8	Butyl benzyl phthalate	BBP	85-68-7
9	Diethylhexyl adipate	DEHA	103-23-1
10	4-Nonylphenol	4-NP	25154-53-3
11	*p*-Octylphenol	p-OP	1806-26-4
12	Bisphenol A	BPA	80-05-7
13	Triphenyltin	TPT	639-58-7
14	Tributyltin	TBT	1461-22-9
15	4-Nitrotoluene	4-NT	99-99-0
16	Benzophenone	BZP	119-61-9
17	Benzo[*a*]pyrene	B[*a*]P	50-32-8
18	Aldicarb		116-06-3
19	Vinclozolin		50471-44-8
20	Carbaryl	NAC	63-25-2
21	Methomyl		16752-77-5
22	Maneb		12427-38-2
23	Mancozeb		8018-01-7
24	Ziram		137-30-4
25	Methoxychlor	MXC	72-43-5
26	Hexachlorocyclohexane	γ-HCH	58-89-9
27	Permethrin		54645-53-1
28	2,4-D		94-75-7
29	2,4,5-T		93-76-5
30	Simazine	CAT	122-34-9
31	Alachlor		15972-60-8
32	PCP		87-86-5
33	Amitrole		61-82-5
34	Nitrofen	NIP	1836-75-5
35	Trifluralin		1582-09-8
36	1,2-dibromo-3-chloropropane	DBCP	96-12-8
37	Malathone		121-75-5
38	Kelthene		115-32-2
39	2,4-Dichlorophenol	DCP	120-83-2
40	Octachlorostyrene	OCS	29082-74-4

(MP Biomedicals, Aurora, OH). Mouse F9 embryonic carcinoma cells were maintained in 5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS) (MP Biomedicals).

Transient Transfection Assays. One day before transfection, 1×10^5 cells were plated in a 35-mm dish containing phenol red-free minimum Eagle's medium (Nissui) supplemented with 10% charcoal/dextran-treated FBS. The cells were transfected by lipofection using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) with pBK-CMV-GAL4-hRXRα or pM-mPPARγ1 (300 ng/dish), p4xUAS-tk-luc (600 ng/dish), and RSV-βgal (100 ng/dish). Fresh medium with or without test chemical was added the day after transfection. After incubation for 24 h, cells were harvested and assayed for luciferase and β-galactosidase activity.

Adipocyte Differentiation Assays. Mouse 3T3-L1 preadipocyte cells were used for the differentiation experiments. The day after the cells reached confluence, the medium was replaced with DMEM containing 10% FBS, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxantine (IBMX), and 1 μM dexamethasone (Dex). At the same time, the cells were treated with a test chemical (rosiglitazone, 9-*cis* retinoic acid, or an organotin compound). After 60 h, the medium was replaced with DMEM containing 10% FBS, 5 μg/ml insulin, and the test chemical. After 6 days, cells were fixed with 4% paraformalde-

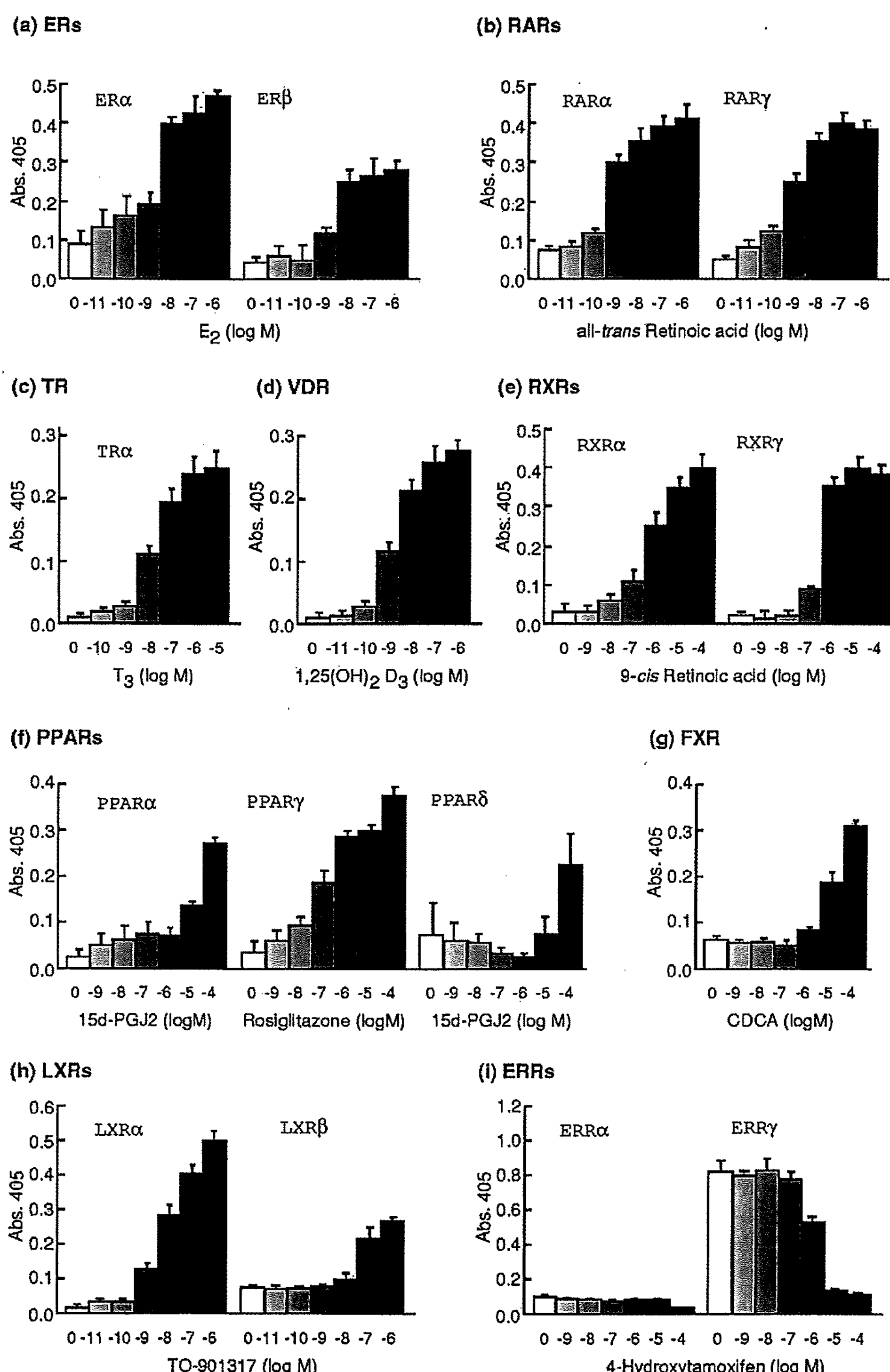

Fig. 1. Ligand-dependent interaction of nuclear receptor and TIF2 in vitro. Ligand-dependent interactions between nuclear receptors and TIF2-BAP were determined as relative alkaline phosphatase activity (vertical axis). The receptor-ligands pairs tested were ERα/β-17β-estradiol (E_2), RARα/γ-all-*trans* retinoic acid, TRα-3,5,3′-triiodo-L-thyronine (T_3), VDR-1α, 25-dihydroxy cholecalciferol [$1,25(OH)_2D_3$], RXRα/γ-9-*cis* retinoic acid, PPARα/δ-15-deoxy-$^{12,14}\Delta$-prostaglandin J_2 (PGJ2), PPARγ-rosiglitazone, LXRα/β-TO-901317, FXR-chenodeoxy cholic acid (CDCA), and ERRα/γ-4-hydroxytamoxifen. Data shown are means ± standard deviation of three independent experiments.