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.

Acknowledgments

This research was supported in part by Grants in Aid for Scientific Research (No. 15201012) from the Ministry of Education, Science, Sports, and Culture of Japan; The Industrial Technology Research Grant Program in 2001 from NEDO (New Energy and Industrial Technology Development Organization of Japan); Health and Labor Sciences Research Grants (Research on Advanced Medical Technology) from the Ministry of Health, Labor, and Welfare of Japan; the fund for endocrine disruption research from the Ministry of the Environment of Japan; and the Long-range Research Initiative (LRI) by Japan Chemical Industry Association (JCIA). We thank Astellas Pharma for providing LG100268.

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Marine Biology (2006) 149: 117–124
DOI 10.1007/s00227-005-0210-3

RESEARCH ARTICLE

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

Received: 15 March 2005 / Accepted: 25 July 2005 / Published online: 15 December 2005

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

Communicated by R. Cattaneo-Vietti, Genova

Physical and Chemical Impacts on Marine Organisms, a Bilateral Seminar Italy–Japan held in November 2004

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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 (Giguére 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,

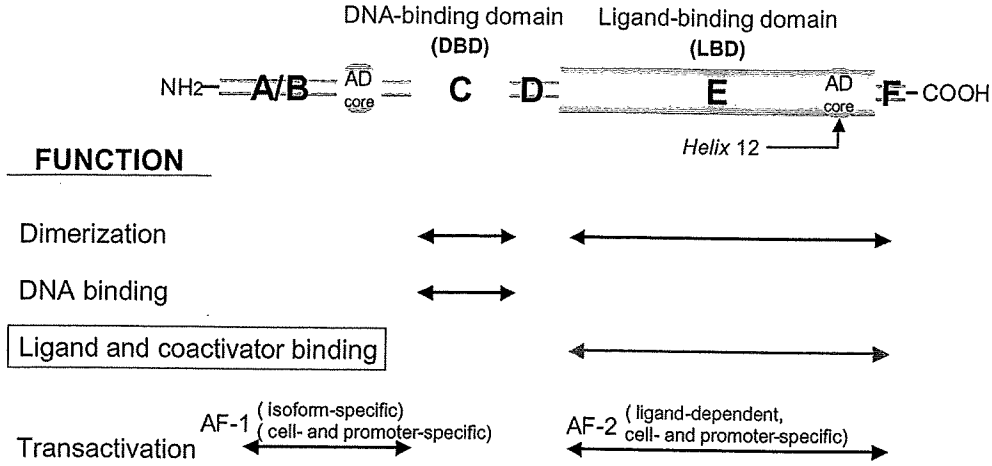

Fig. 1 Typical structure and functional domain of a nuclear receptor. Nuclear receptors are highly structurally related and share a common structural organization with a variable amino-terminal domain (**a/b**); a central, well-conserved DNA-binding domain (**c**); a non-conserved hinge domain (**d**); 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

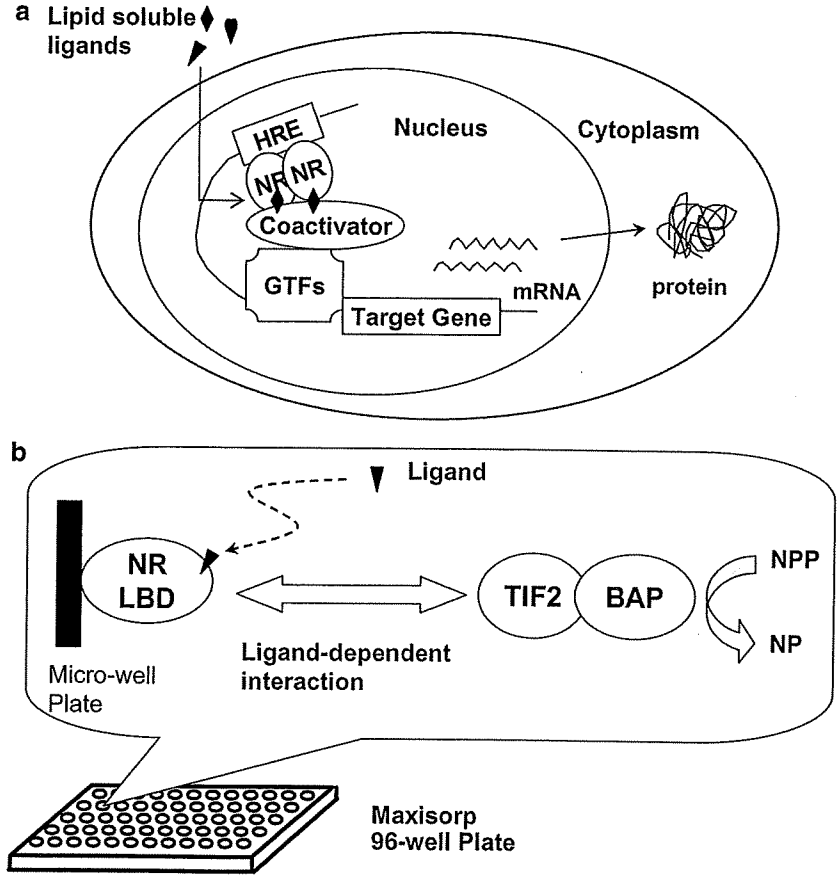

Fig. 2 **a** Nuclear receptors act as ligand-activated transcription factors by directly interacting with DNA-response elements of target genes as homodimers, heterodimers, or monomers. The effects of nuclear receptors on transcription are mediated through recruitment of co-regulators. Upon ligand binding, 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).

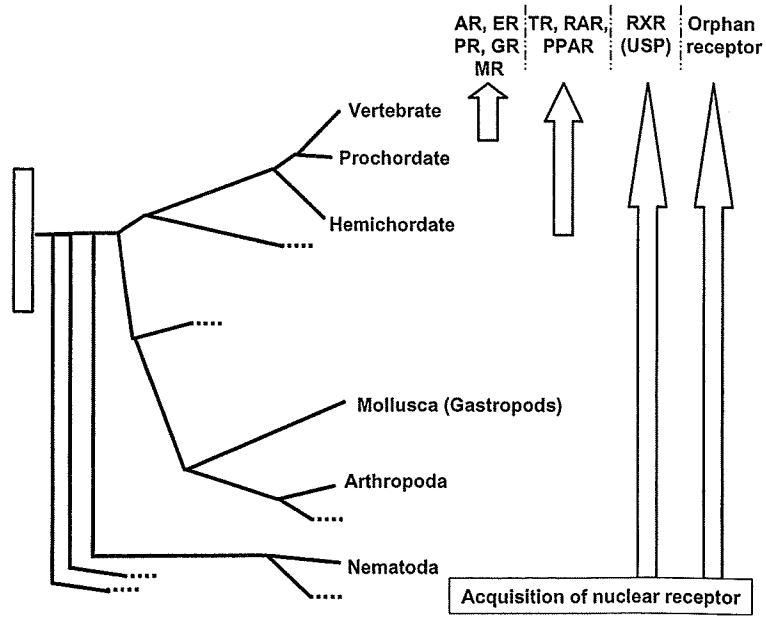

Fig. 3 Evolutionary tree and acquisition of nuclear receptors. Steroid hormone receptors (*AR* androgen receptor, *ER* estrogen receptor, *PR* progesterone receptor, *GR* glucocorticoid receptor, *MR* mineralocorticoid receptor) exist only in vertebrates. The subfamily of TR, RAR, and PPAR are present 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)

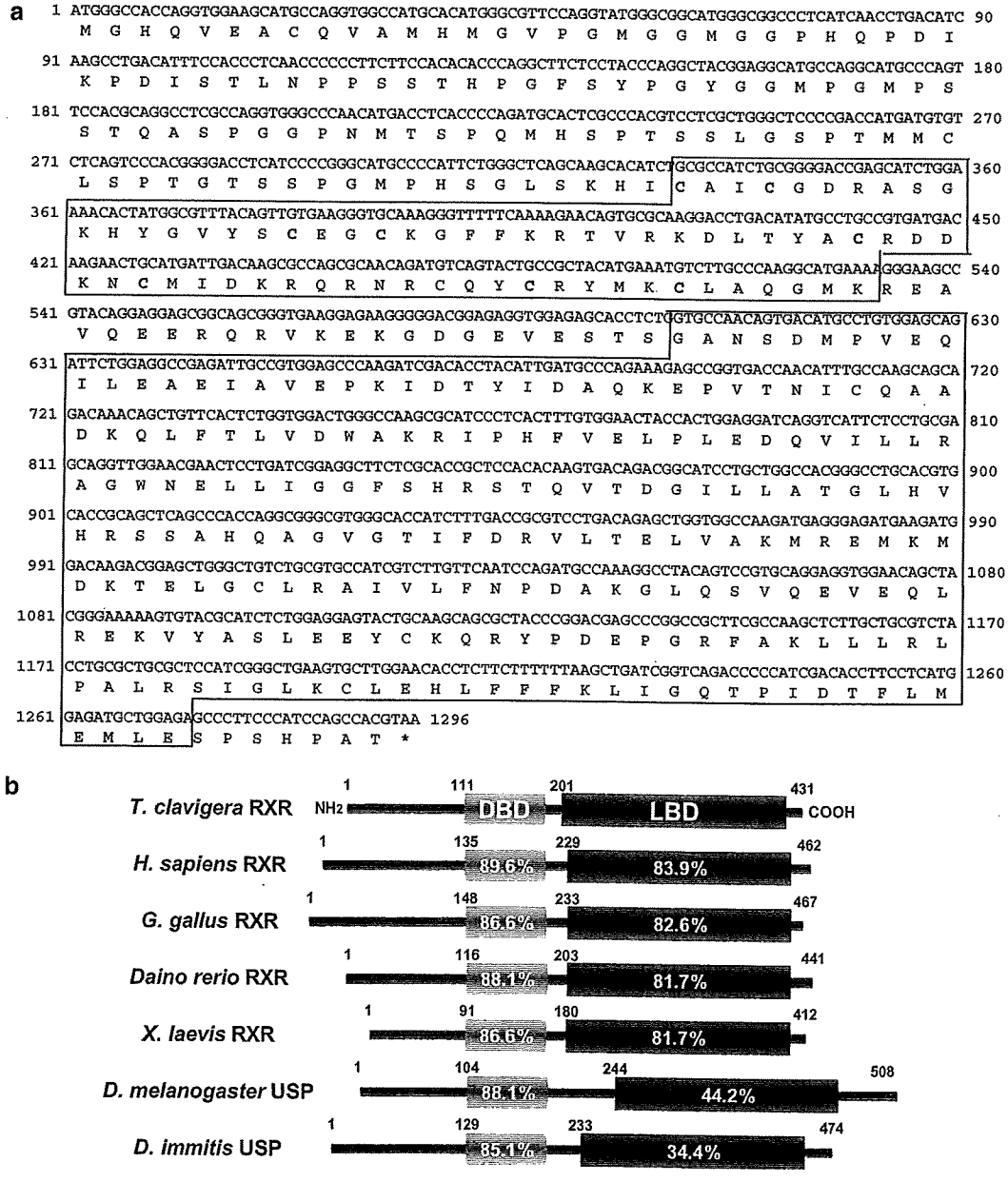

Fig. 4 a The entire coding sequence of gastropod RXR. The DNA and LBDs are *boxed*. The database accession number for the sequence is AY704160. b A schematic representation of RXR from various species is shown, along with the percentage of identical amino acid residues shared with those of gastropod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

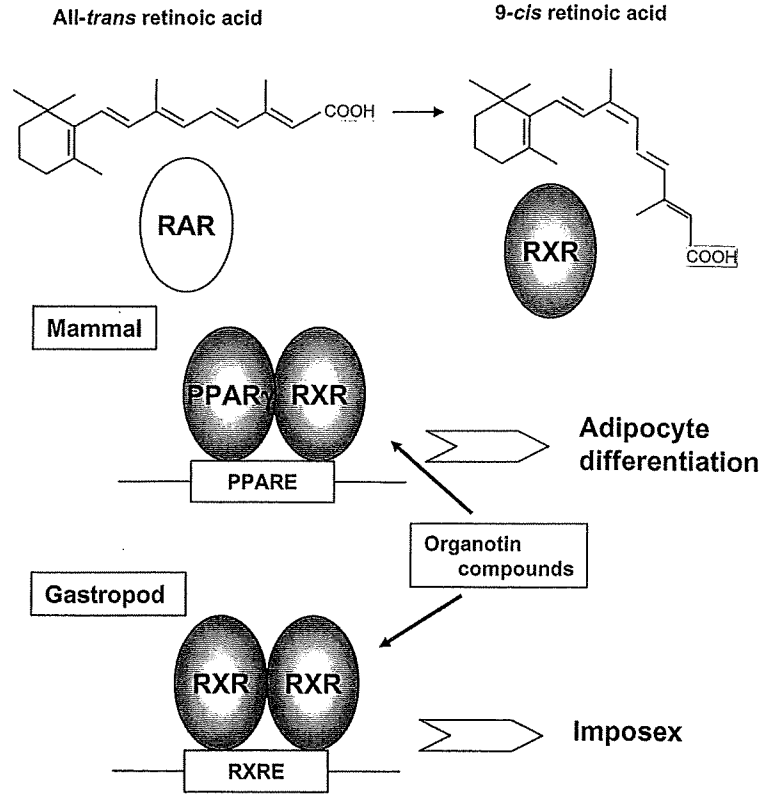

Fig. 5 Effects of organotin compounds in mammals and gastropods via RXR. In mammals, organotin compounds are ligands for both PPARγ and RXR and affect adipocyte differentiation. In gastropods, organotin compounds induce imposex by binding to RXR

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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0026-895X/05/6703-766–774$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 67:766–774, 2005

Vol. 67, No. 3
8409/1196795
Printed in U.S.A.

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

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Received October 18, 2004; accepted December 20, 2004

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.

This work was supported by grants from the Ministry of the Environment, and Health, Labor, and Welfare, Japan.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.008409.

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-

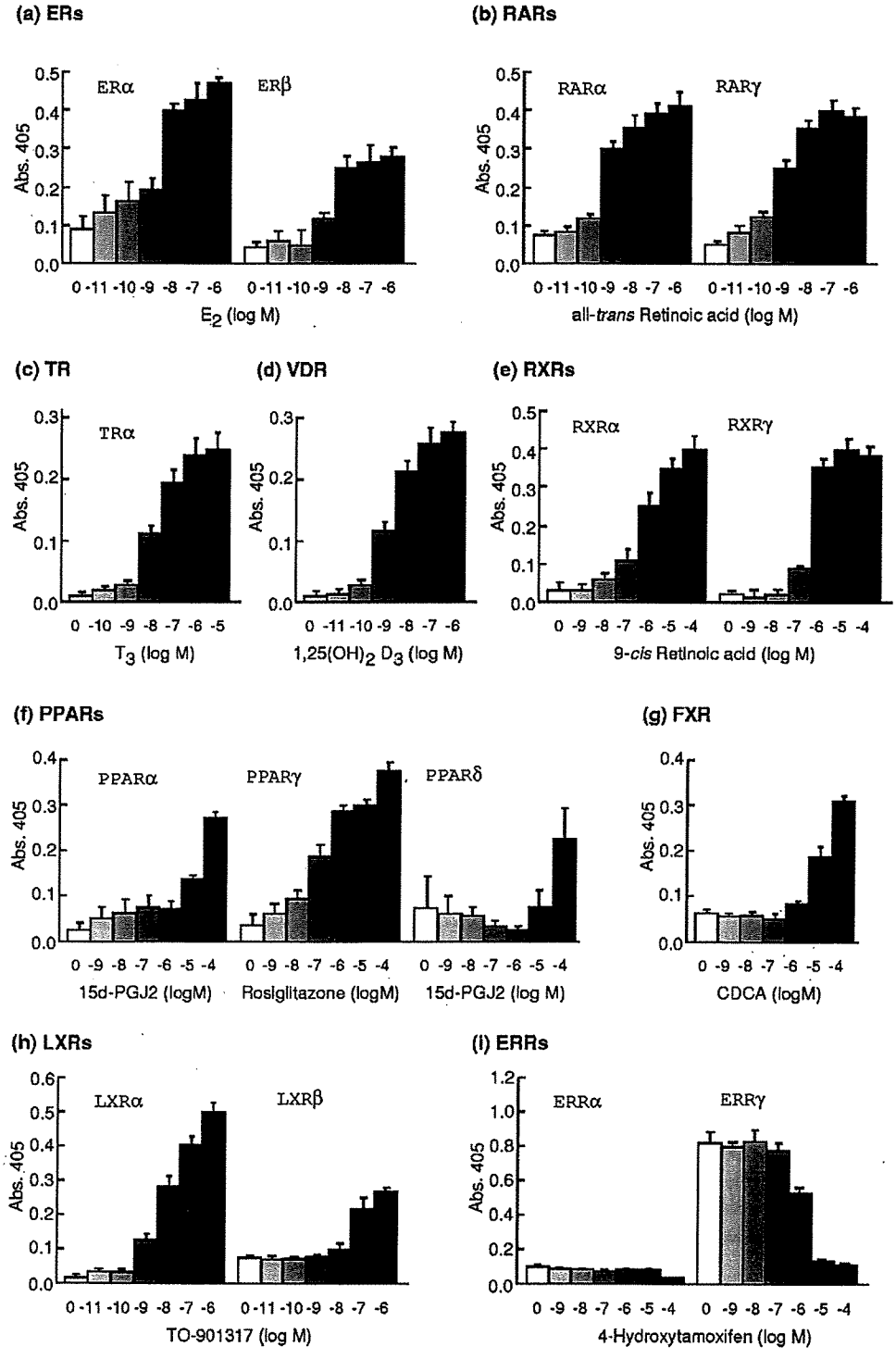

Fig. 1. Ligand-dependent interaction of nuclear receptor and TIF2 in vitro. Ligand-dependent interactions between nuclear receptors and TIF2-BAP were determined as relative alkaline phosphatase activity (vertical axis). The receptor-ligands pairs tested were ERα/β-17β-estradiol (E_2), RARα/γ-all-*trans* 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}Δ-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.

hyde and stained with 0.5% Oil Red O. The amount of triglyceride was determined by Triglyceride E Test (Wako Pure Chemicals).

RNA Isolation, Northern Blotting, and RT-PCR Analyses. The 3T3-L1 cells were grown in DMEM containing 10% calf serum. The day after the cells became confluent, they were treated with vehicle (dimethyl sulfoxide) only, rosiglitazone (Rosi), TPT, or TBT in DMEM containing 10% FBS and 5 μg/ml insulin. The cells were harvested at various times after treatment, and total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). For Northern blot analyses, 25 μg of total RNA was electrophoresed through a 1% agarose gel containing 2% formaldehyde and then transferred to a Hibond-N^+ nylon membrane (Amersham Biosciences Inc.). The filter was hybridized with each probe, which was labeled with [α-^{32}P]dCTP by using a random labeling kit (TaKaRa, Shiga, Japan). For RT-PCR, cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan), and polymerase chain reaction was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA). The primers used for amplification of the aP2 gene (a marker for adipocyte differentiation) were 5′-AAAATGTGTGATGCCTTTGTGGG-3′ and 5′-TCATGCCCTTTCATAAACTCTTGTGG-3′.

Results

Application of CoA-BAP System to Endocrine Disruptors. Reproductive abnormalities in wildlife can be associated with exposure to environmental pollutants capable of mimicking the action of natural hormones. Because the nuclear receptors of intrinsic hormone systems are likely to be targets of industrial chemicals, information on their ability to bind these chemicals is valuable for environmental risk assessment. To determine whether suspected endocrine disruptors can bind to members of the nuclear receptor family, we constructed assay systems for human nuclear receptors, including ERα/β, RARα/γ, TRα, VDR, RXRα/γ, PPARα/γ/δ, FXR, LXRα/β, and ERRα/γ, on the basis of the previously described CoA-BAP system (Kanayama et al., 2003). The cognate ligand for each nuclear receptor enhanced alkaline phosphatase activity in a dose-dependent manner (Fig. 1). In the ERR systems, 4-hydroxy tamoxifen-dependent dissociations between ERR and coactivator were observed, as reported previously (Coward et al., 2001; Tremblay et al., 2001).

Using these systems, we evaluated 40 suspected endocrine disruptors (Table 1) recognized by various organizations (e.g., World Health Organization and Ministry of the Environment in Japan). The effects of the tested chemicals on the interaction between nuclear receptors and TIF2 (Fig. 2) suggest that several compounds possess agonistic activities for multiple receptors simultaneously. Butyl benzyl phthalate, hexachlorocyclohexane, maneb, mancozeb, and alkylphenols were weakly agonistic for multiple receptors, including ER. One intriguing finding was that the effect of TBT on RXRα was as strong as that of its endogenous ligand, 9-*cis* retinoic acid (Fig. 3), and the agonist effect of TPT on PPARγ was as strong as that of its well known ligand, Rosi (Fig. 3). The EC_{50} values of TBT on RXRα (7.4×10^{-8} M) and TPT on PPARγ (9.5×10^{-8} M) were almost the same as those of 9-*cis* retinoic acid (4.3×10^{-8} M) and Rosi (1.1×10^{-7} M), respectively. Because triphenylmethane and triphenylethylene were not agonistic for RXRα and PPARγ, the tin moiety was important for activity (Fig. 3).

Organotin Compounds Potentiated Transactivation by RXR and PPARγ. The observations that organotin compounds enhanced the protein-protein interaction between the coactivator TIF2 and RXRα or PPARγ suggested that these compounds activate transcription via these receptors. To confirm the results we obtained from the CoA-BAP system, we performed a reporter gene assay in mammalian culture cells using an expression vector for (GAL4-DBD)-RXRα or (GAL4-DBD)-PPARγ and a reporter plasmid containing the luciferase gene along with GAL4 upstream activating sequence. Both TPT and TBT induced the transactivation function of RXRα or PPARγ in a dose-dependent manner (Fig. 4). The effectiveness of these organotin compounds was comparable with that of known ligands. In addition, dibutyltin chloride, a TBT metabolite in vivo, also activated reporter activity in the PPARγ system (data not shown).

Induction and Promotion of Adipocyte Differentiation by Organotin Compounds in 3T3-L1 Cells. Recent studies indicate that PPARγ plays a central role in adipocyte gene expression and differentiation (Tontonoz et al., 1994). 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). If or-

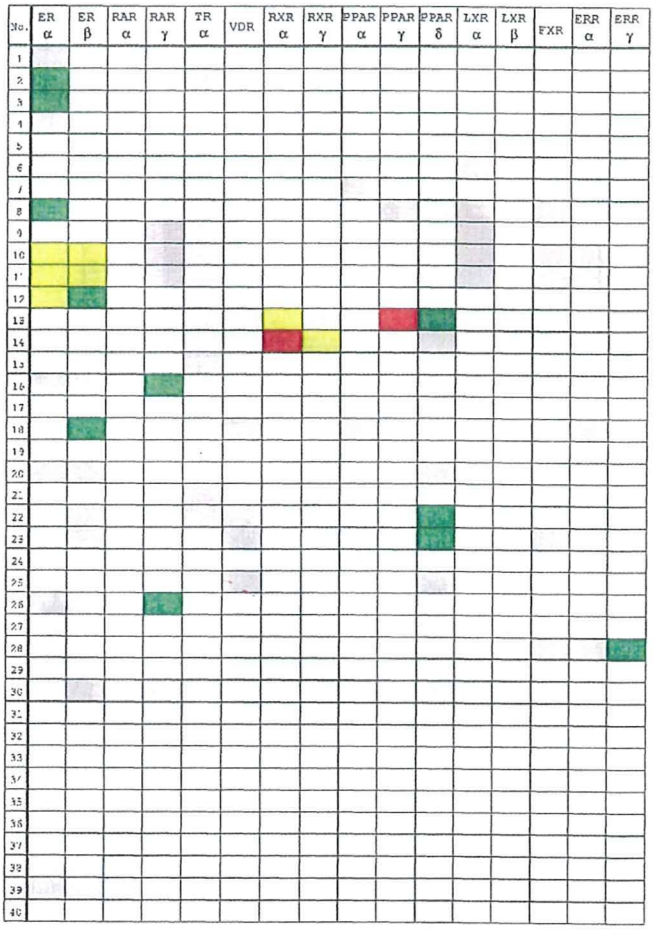

Fig. 2. Agonistic activities of suspected endocrine disruptors for various nuclear receptors. The effects of chemicals on the interaction between nuclear receptors and the coactivator TIF2 were assessed using the CoA-BAP system. The numbers in the far left column correspond to the chemicals listed in Table 1. The lowest effective concentrations of test chemicals were determined and compared with lowest effective concentration of cognate ligands shown in Fig. 1: red, ~1 to 10 times as much as cognate ligand; yellow, ~10 to 100; green, ~100 to 1000; gray, ~1000 to 10,000 times; and white, not detected. Triphenyltin (13) and tributyltin (14) showed strong activity on PPARγ and RXRα, respectively.

ganotin compounds can function as activators for PPARγ/RXR in vivo, these compounds probably induce adipocyte differentiation. To investigate this possibility, we treated 3T3-L1 cells with TPT or TBT in two types of differentiation medium, a complete differentiation medium that contained the inducers IBMX, Dex, insulin, and FBS and an incomplete differentiation medium that lacked IBMX and Dex. Although insulin is not always necessary for induction of differentiation, it efficiently enhances adipocyte development. Adipocyte differentiation was confirmed by staining with Oil Red O for lipid droplet accumulation. As expected, treatment of 3T3-L1 cells with either TPT or TBT in complete differentiation medium promoted adipocyte differentiation as well as did Rosi (Fig. 5, a–d). Even in incomplete differentiation medium, addition of organotin compounds induced adipocyte differentiation in contrast with the lack of induction after treatment with vehicle only (Fig. 5, e–h). Moreover, mRNA expression of the adipocyte differentiation marker aP2 was induced in a dose-dependent manner by addition of organotin compounds (Fig. 6a). PPARγ mRNA also was induced during the differentiation process (Fig. 6a), in agreement with the results of a previous study (Tontonoz et al., 1994). Induction of aP2 mRNA expression occurred late in adipogenesis (Fig. 6b), and organotin-treated cells demonstrated accumulation of triglyceride (Fig. 6c). Together, these data provide strong evidence that the organotin compounds TPT and TBT can function as inducers of adipocyte differentiation through PPARγ.

Discussion

Our study was designed to evaluate the effects of suspected endocrine disruptors on various nuclear receptors. The data show that several compounds have simultaneous effects on multiple nuclear receptors. In particular, organotin compounds (e.g., TBT and TPT) showed strong effects on RXR or PPARγ, at levels comparable with those of 9-*cis* retinoic acid, an endogenous RXR ligand, and rosiglitazone, a known agonist of PPARγ. In CoA-BAP systems, TBT showed strong effect on protein-protein interaction between RXRα and TIF2, but TPT showed slight effect (Fig. 3a). TPT showed strong effect on protein-protein interaction between PPARγ

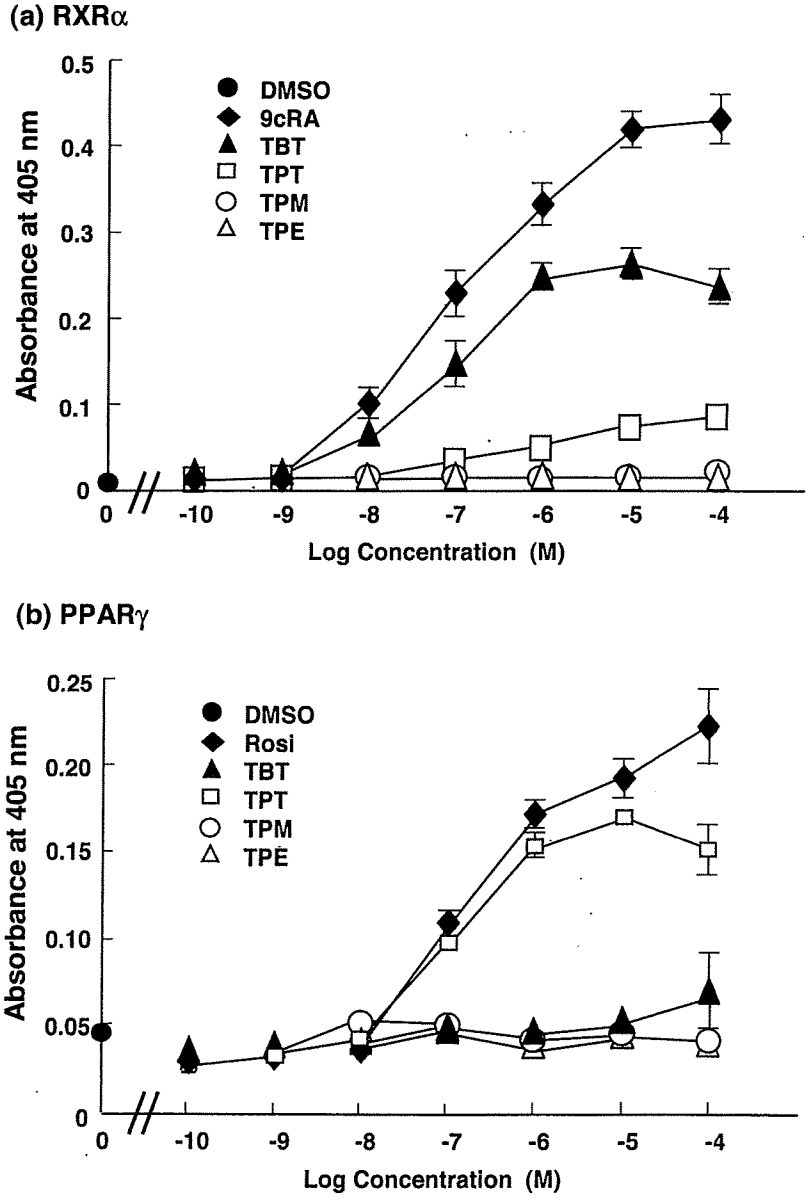

Fig. 3. Dose-response curves of the effects of organotin compounds on hRXRα and human PPARγ (hPPARγ) in the CoA-BAP system. A, TBT (▲) showed strong agonistic activity for hRXRα at as low a concentration as that of 9-*cis* retinoic acid (9cRA, ♦). B, TPT (□) showed strong agonistic activity to hPPARγ at as low a concentration as that of Rosi (♦). TPM (○) and TPE (△) did not show any agonistic activity. Activity of the vehicle control (dimethyl sulfoxide) only is shown by ●.

and TIF2, but TBT did not (Fig. 3b). On the contrary, when tested in the transactivation assay, both TBT and TPT activated not only RXRα but also PPARγ (Fig. 4). This discrepancy might reflect the diversity of coactivators. To date, many coactivators have been identified as nuclear receptor-interacting proteins. These coactivators are supposed to have cell- or tissue-specific functions in vivo (Smith and O'Malley, 2004). In addition, PPARγ reportedly changes its interaction partners depending on ligands (Kodera et al., 2000). We used only TIF2 in CoA-BAP system, whereas cells used for transactivation assays have many coactivators. The discrepancy of results from CoA-BAP systems and transactivation assays might be explained by this difference of coactivators. Because in vitro screening methods tend to produce false positive or false negative results like this, positive compounds should be further examined by other studies in a physiological context. Therefore, we examined the effects of organotin compounds on transcriptional regulation and adipogenesis, which is a famous physiological event related to PPARγ/RXR pathway.

Exposure of rats in utero to TBT induces a dramatic increase in the incidence of low-birth-weight fetuses because of maternal hypothyroidism (Adeeko et al., 2003). Furthermore,

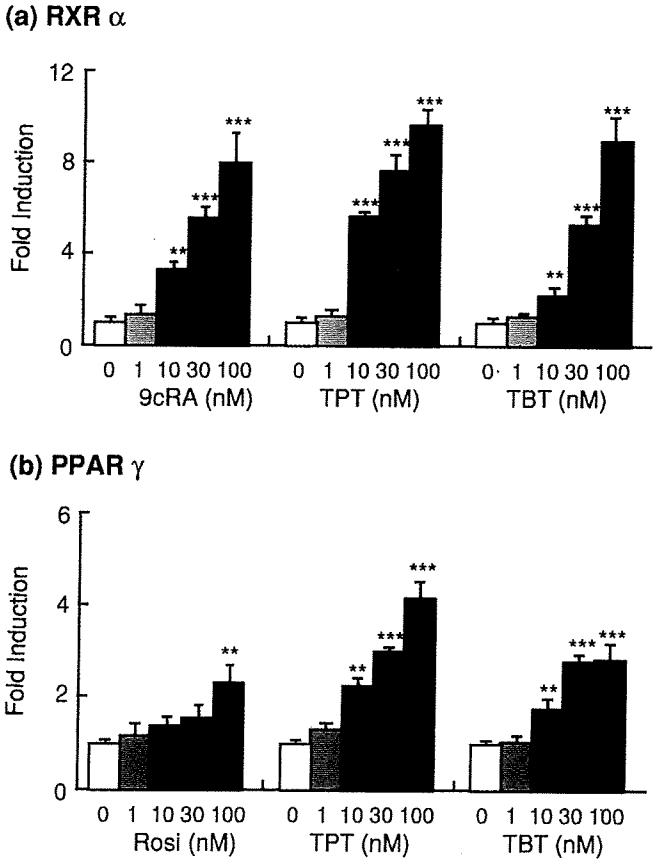

Fig. 4. Organotin compounds induce transcriptional activity through RXRα and PPARγ. Ligand-dependent transactivation of RXRα and PPARγ were detected as luciferase activity. a, F9 cells were cotransfected with a GAL4-DBD-hRXRα expression plasmid and a GAL4-responsive reporter plasmid. b, NIH-3T3 cells were cotransfected with a GAL4-DBD-mPPARγ1 expression plasmid and a GAL4-responsive reporter plasmid. The luciferase activities relative to the β-galactosidase activity are shown and represent the fold-stimulation compared with the activity of the vehicle-only control. Data shown are the means ± standard deviation of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ significantly different from vehicle controls.

the RXR agonist bexarotene causes clinically significant hypothyroidism in patients with cutaneous T-cell lymphoma (Duvic et al., 2001), and experimental exposure of rats to LG100268 (a selective RXR agonist) induces the acute phase of hypothyroidism (Liu et al., 2002). The similarities between the toxicities of TBT and selective RXR agonists suggested to us that at least some of the toxic effects of organotin compounds are mediated by RXR.

Most of the toxic effects of organotin compounds on sexual development and reproductive function have been documented in mollusks (Matthiessen and Gibbs, 1998). In gastropods, TBT and TPT cause imposex (Morcillo and Porte, 1999), an irreversible syndrome in which male genital tracts (mainly a penis and a vas deferens) are imposed on female organisms (Smith, 1971). Although the physiological functions of organotin compounds have been studied extensively, the molecular target of organotin compounds had been unclear. To this end, we found that TPT and TBT were agonists for RXR and PPARγ. It has been thought that the sexual toxicity of organotin compounds results from increased androgen levels because of inhibition of the aromatase enzyme complex that catalyzes conversion of androgen to estrogen. This enzyme complex consists of microsomal CYP19 and the reduced form of the flavoprotein nicotinamide adenine dinucleotide phosphate reductase. TBT-induced imposex in neogastropods reportedly is mediated by inhibition of aromatase (Bettin et al., 1996), and TBT inhibits the catalytic activity of aromatase derived from transfected cells (Heidrich et al., 2001; Cooke, 2002). However, the effective concentrations of enzyme inhibition were relatively high (above 10^{-6} M). In this study, we found that TBT and TPT induced the transactivation function of RXRα and PPARγ at 10^{-8} M. It is reasonable that the effective concentration on gene expression was different from that on enzyme inhibition. In consistent with this, Nakanishi et al. (2004) demonstrated that 10^{-8} M TBT or TPT induced hCG or aromatase activity along with mRNA expression in placental cells (Nakanishi et al., 2002). In ovarian granulose cells, 20 ng/ml (about 6×10^{-8} M) TBT or TPT suppresses the $P450_{aroma}$ gene expression (Saitoh et al., 2001). We have to consider the toxicities of organotin compounds in distinguishing the low-dose effect from high-dose effect. Recently, we reported that RXR plays an important role in the development of gastropod imposex, by showing the cloning of RXR homolog from marine gastropod, binding of organotins to that receptor, and imposex induction by injection of RXR ligand 9-*cis* retinoic acid (Nishikawa et al., 2004). Gastropod imposex is known to be typically induced by very low concentrations of TBT and/or TPT (Bryan et al., 1986; Gibbs and Bryan, 1986; Horiguchi et al., 1997). Although it has been theorized that organotins increases androgen levels through inhibition of aromatase activity and/or a suppression of androgen excretion, the inhibitory concentration of organotins is not low enough for explaining imposex induction. The low-dose effects are likely to be mediated by receptors. However, the study of organotin effects in mammals is still important, because the compositions of nuclear receptor family members are very different between vertebrates and invertebrates (Escriva et al., 1997; Laudet, 1997). For example, there are no known homologs of steroid hormone receptors in the *Drosophila melanogaster* or *Caenorhabditis elegans* genomes, and the group members of TR, RAR, VDR, and PPAR seem to be late acquisitions dur-

ing the evolution of the superfamily. Therefore, we examined the effects of suspected endocrine disruptors on human nuclear receptor family members. As a result, PPARγ was identified as a new target molecule of organotin compounds in addition to RXR. This finding might introduce new insights in physiological functions of organotin compounds in mammals.

We were surprised to find that organotin compounds were high-affinity ligands for RXR and PPARγ. Until recently, it had been thought that among synthetic compounds, only hormone analogs could bind hormone receptors, because the relationships between hormones and their cognate receptors are very specific. However, some industrial chemicals do have unexpected effects on hormone receptors. Nuclear receptors are the likely targets, because their intrinsic ligands are fat-soluble, low-molecular-weight agents, as are the environmental pollutants. In fact, organotin compounds promote the adipocyte differentiation as agonists for PPARγ/

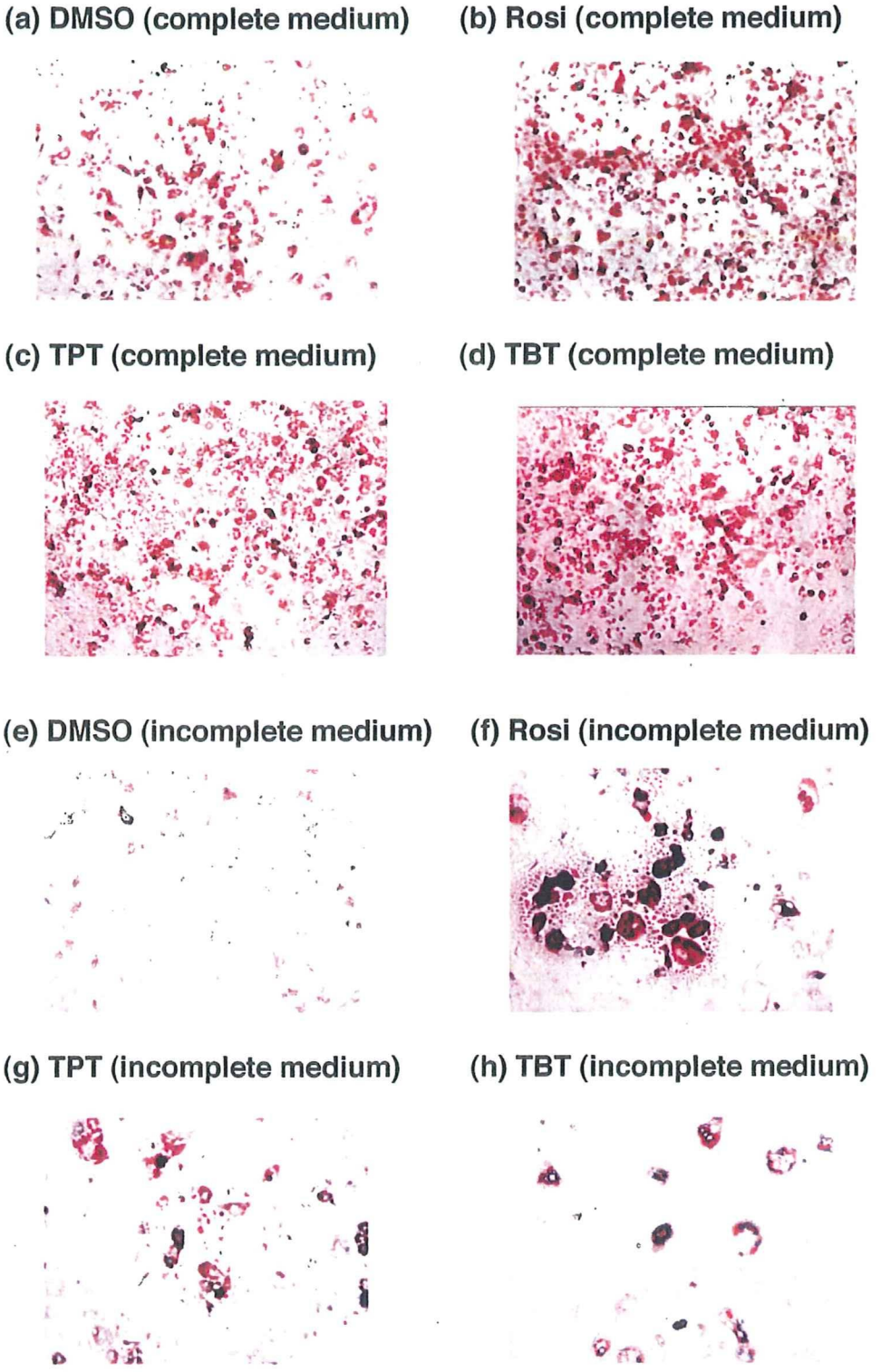

Fig. 5. Enhancement of lipid accumulation by organotin compounds. 3T3-L1 cells were maintained in DMEM containing 10% calf serum. One day after reaching confluence, the cells were treated for 60 h with vehicle only (a and e), 100 nM rosiglitazone (b and f), 100 nM TPT (c and g), or 100 nM TBT (d and h) in complete differentiation medium (a–d) or incomplete differentiation medium (e–h). The cells received fresh medium every 48 h. On the 10th day after induction of differentiation, the cells were fixed with paraformaldehyde and stained with Oil Red O.

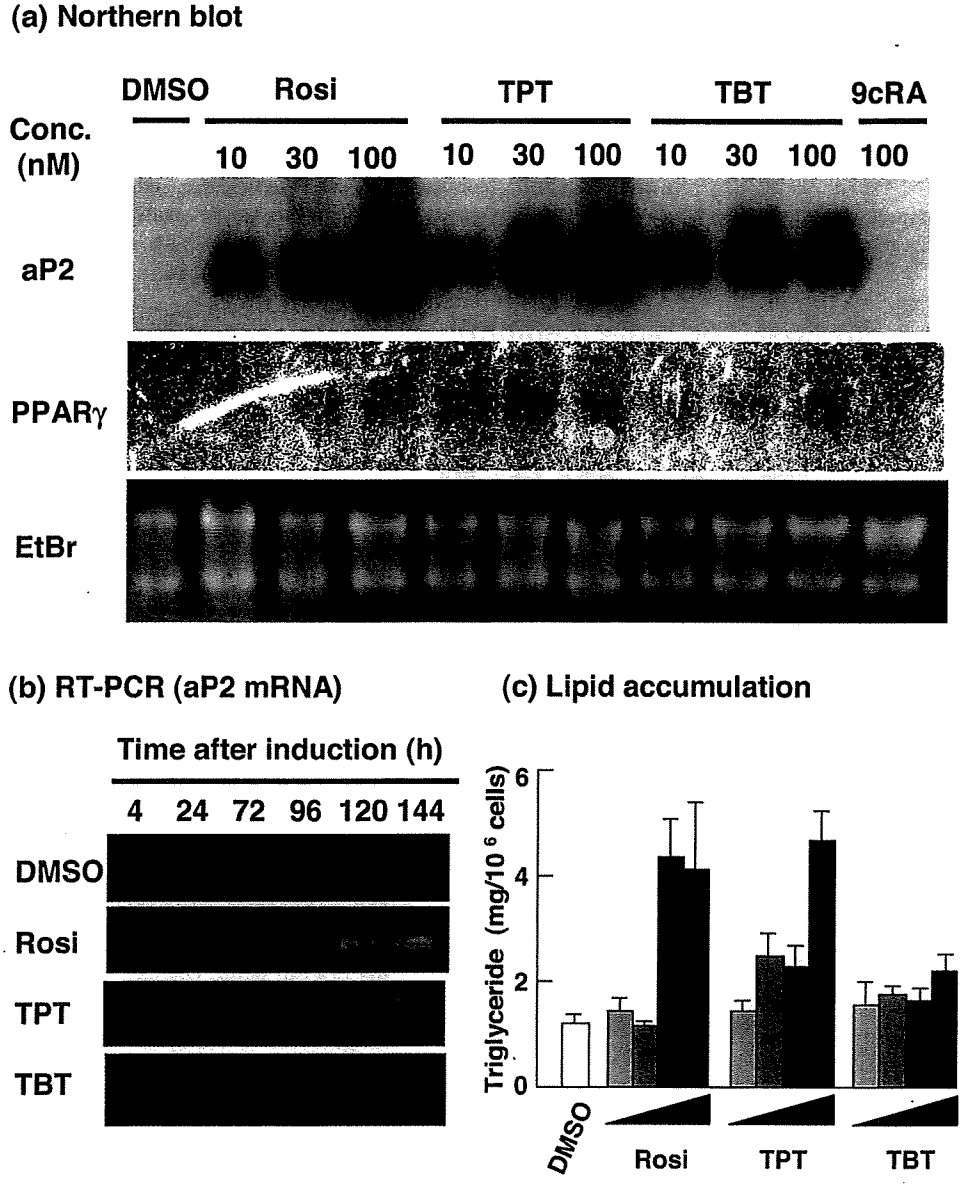

Fig. 6. Induction of adipocyte differentiation markers by organotin compounds. a, induction of adipocyte marker genes by organotin compounds in incomplete differentiation medium. 3T3-L1 cells were maintained in DMEM containing calf serum. One day after reaching confluence, the cells were treated with vehicle only, rosiglitazone (10–30 nM), TPT (10–30 nM), TBT (10–30 nM), or 9-*cis* retinoic acid (100 nM) in DMEM containing 10% FBS and 10 μg/ml insulin. Total RNA was isolated at 10 days after treatment, and mRNA expression of the aP2 and PPARγ genes was detected by Northern blot analysis. The ethidium bromide staining for ribosomal RNAs is shown as a control. b, time course of aP2 gene expression. 3T3-L1 cells were treated with vehicle only, rosiglitazone (100 nM), TPT (100 nM), or TBT (100 nM) in incomplete differentiation medium. The cells were harvested at the indicated time after treatment, and mRNA expression of the aP2 gene was analyzed by RT-PCR. c, lipid accumulation in differentiated 3T3-L1 cells. The cells were treated with 1, 10, 30, or 100 nM chemical. Ten days later, the amount of triglyceride was determined as described under *Materials and Methods*.

RXR. The ligands of PPARγ and RXR are expected for antidiabetic agents, but they have some side effects at the same time (Mukherjee et al., 1997; Yaki-Jarvinen, 2004). Although they may be good medicines when used under a doctor's control, wildlife are exposed to synthetic chemicals in uncontrolled manner. It is possible that TBT and TPT cause adverse health effects on the organisms by disturbing the endocrine process mediated by PPARγ/RXR.

Acknowledgments

We are grateful to Dr. Y. Kamei (National Institute of Health and Nutrition, Japan) for providing the GAL4-reponsive reporter plasmid and pM-mPPARγ1.

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Some organotin compounds enhance histone acetyltransferase activity

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Received 16 September 2004; received in revised form 24 October 2004; accepted 25 October 2004
Available online 28 November 2004

Abstract

Eukaryotic DNA is packaged into chromatin, whose basic subunit is the nucleosome, which consists of DNA and a core histone octamer. Histone acetylation is important for the regulation of gene expression and is catalyzed by histone acetyltransferase (HAT). We observed the effects of suspected endocrine-disrupting chemicals (EDCs) on HAT activity. We showed that some organotin compounds – tributyltin (TBT) and triphenyltin (TPT) – enhanced HAT activity of core histones in a dose-dependent way and other EDCs did not affect HAT activity. Organotin compounds have various influences on physical function including the hormone and immune systems, embryogenesis, and development. Dibutyltin and diphenyltin, metabolites of TBT and TPT, respectively, also promoted HAT activity, but monobutyltin, monophenyltin, and inorganic tin had no effect. Further, TBT and TPT enhanced HAT activity when nucleosomal histones were used as substrates. These data indicate that the organotin compounds have unique effects on HATs independent of their EDC activities and suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

Keywords: Histone acetyltransferase; Organotin; Tributyltin; Triphenyltin; Endocrine-disrupting chemical

1. Introduction

Nuclear eukaryotic DNA is packaged into chromatin, which has a major impact on levels of gene transcription. The basic unit of chromatin is the nucleosome core particle, which consists of 146 bp of DNA wrapped around a histone octamer. This octamer con-

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doi:10.1016/j.toxlet.2004.10.009