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).

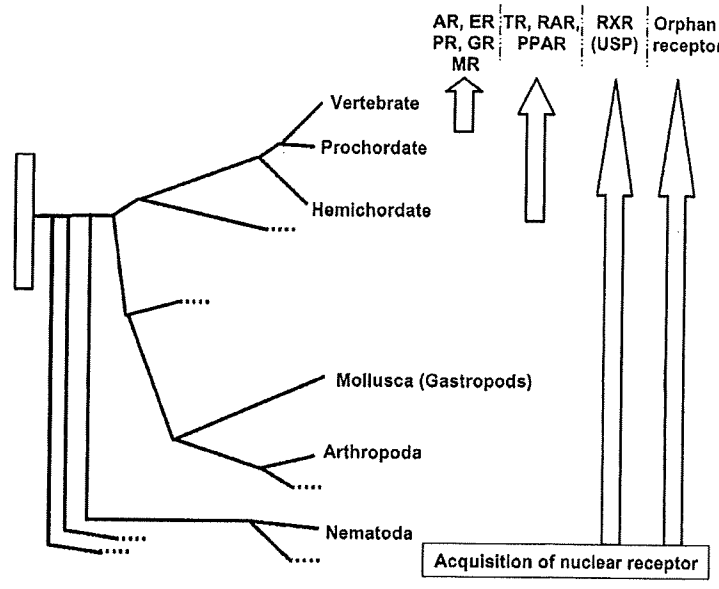

Fig. 3 Evolutionary tree and acquisition of nuclear receptors. Steroid hormone receptors (*AR* androgen receptor, *ER* estrogen receptor, *PR* progesterone receptor, *GR* glucocorticoid receptor, *MR* mineralocorticoid receptor) exist only in vertebrates. The subfamily of TR, RAR, and PPAR are present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

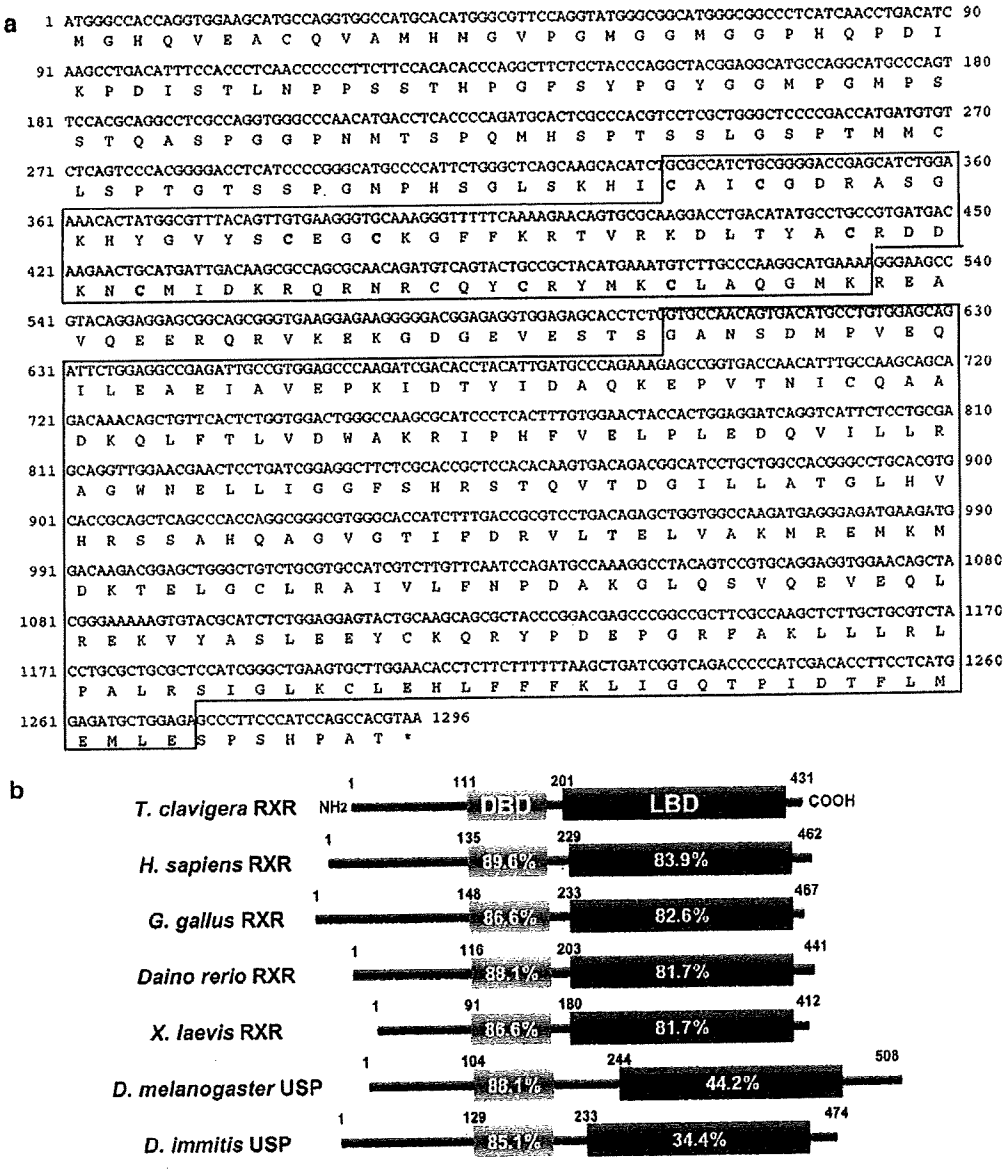

Fig. 4 a The entire coding sequence of gastropod RXR. The DNA and LBDs are *boxed*. The database accession number for the sequence is AY704160. b A schematic representation of RXR from various species is shown, along with the percentage of identical amino acid residues shared with those of gastropod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

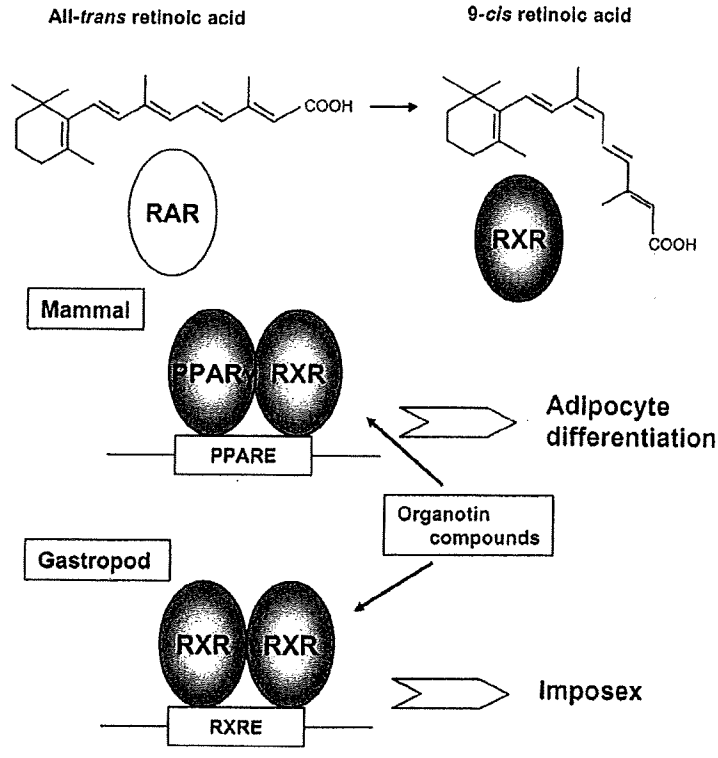

Fig. 5 Effects of organotin compounds in mammals and gastropods via RXR. In mammals, organotin compounds are ligands for both PPARγ and RXR and affect adipocyte differentiation. In gastropods, organotin compounds induce imposex by binding to RXR

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.

References

Agostini M, Fletterick RJ, Beck-Peccoz P, Reinhardt W, Binder G, Ranke MB, Hermus A, Hesch RD, Lazarus J, Newrick P, Parfitt V, Raggatt P, Zegher FD, Chatterjee VKK (1998) A role for helix 3 of the TRβ ligand-binding domain in coactivator recruitment identified by characterization of a third cluster of mutations in resistance to thyroid hormone. EMBO J 17:4760–4770

Axiak V, Vella AJ, Micaleff D, Chircop P, Mintoff B (1995) Imposex in *Hexaplex trunculus* (Gastropoda: Muricidae): first results from biomonitoring of tributyltin contamination in the Mediterranean. Mar Biol 121:685–691

Bettin C, Oehlmann J, Stroben E (1996) TBT-induced imposex in marine neo-gastropods is mediated by an increasing androgen level. Helgolander Meeresunters 50:299–317

Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 54:138–153

Boehm MF, Zhang L, Zhi L, McClurg MR, Berger E, Wagoner M, Mais DE, Suto CM, Davies JA, Heyman RA (1995) Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. J Med Chem 38:3146–3155

Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. Nature 375:377–382

Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom L, Greene GL, Gustagsson JA, Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758

Bryan GW, Gibbs PE, Burt GR (1988) A comparison of the effectiveness of tri-n-butyltin chloride and five other organotin compounds in promoting the development of imposex in the dog-whelk, *Nucella lapillus*. J Mar Biol Assoc UK 68:733–744

Bryan GW, Gibbs PE, Burt GR, Hummerstone LG (1987) The effects of tributyltin (TBT) accumulation on adult dog-whelk, *Nucella lapillus*: long-term field and laboratory experiments. J Mar Biol Assoc UK 67:525–544

Bryan GW, Gibbs PE, Hummerstone LG, Burt GR (1986) The decline of the gastropod *Nucella lapillus* around the south-west of England: evidence for the effect of tributyltin from antifouling paints. J Mar Biol Assoc UK 66:611–640

Colborn T, Dumanoski D, Myers JP (1996) Our stolen future. Dutton, New York

Cooke GM (2002) Effects of organotins on human aromatase activity in vitro. Toxicol Lett 126:121–130

Escriva H, Delaunay F, Laudet V (2000) Ligand binding and nuclear receptor evolution. BioEssays 22:717–727

Escriva H, Safi R, Hanni C, Langlois M-C, Saumitou-Laprade P, Sthehelin D, Capron A, Pierce R, Laudet V (1997) Ligand binding was acquired during evolution of nuclear receptors. Proc Natl Acad Sci USA 94:6803–6808

Fent K (1996) Ecotoxicology of organotin compounds. Crit Rev Toxicol 26:1–117

Féral C, Le Gall S (1983) The influence of a pollutant factor (tributyltin) on the neuroendocrine mechanism responsible for the occurrence of a penis in the females of Ocenebra erinacea. In: Lever J, Boer HH (eds) Molluscan neuro-endocrinology. North Holland Publ Co, Amsterdam, pp 173–175

Gibbs PE, Bryan GW (1986) Reproductive failure in populations of the dog-whelk, *Nucella lapillus*, caused by imposex induced by tributyltin from antifouling paints. J Mar Biol Assoc UK 66:767–777

Gibbs PE, Bryan GW, Pascoe PL, Burt GR (1987) The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. J Mar Biol Assoc UK 67:507–523

Giguére V (1999) Orphan nuclear receptors: from gene to function. Endocr Rev 20:689–725

Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Oriando E, Guillette L (2001) Effects of environmental antiandrogens on reproductive development in experimental animals. Hum Reprod Update 7:248–264

Heidrich DD, Steckelbroek S, Klingmuller D (2001) Inhibition of human cytochrome P450 aromatase activity by butyltins. Steroids 66:763–769

Henrich VC, Brown NE (1995) Insect nuclear receptors: a developmental and comparative perspective. Insect Biochem Mol Biol 25:881–897

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 68:397–406

Horiguchi T, Shiraishi H, Shimizu M, Morita M (1997a) Imposex in sea snails, caused by organotin (tributyltin and triphenyltin) pollution in Japan: a survey. Appl Organomet Chem 11:452–455

Horiguchi T, Shiraishi H, Shimizu M, Morita M (1997b) Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell, *Thais clavigera*. Environ Pollut 95:452–455

Horton C, Maden M (1995) Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. Dev Dyn 202:312–323

Kanayama T, Kobayashi N, Mamiya S, Nakanishi T, Nishikawa J (2005) Organotin compounds promote adipocyte differentiation as agonists of the peroxisome activated receptor (PPAR)γ/retinoid X receptor (RXR) pathway. Mol Pharmacol 67:766–774

Kanayama T, Mamiya S, Nishihara T, Nishikawa J (2003) Basis of a high-throughput method for nuclear receptor ligand. J Biochem 133:791–797

Kastner P, Grondona J, Mark M, Gansmuller A, LeMeur M, Decimo D, Vonesch JL, Dolle P, Chambon P (1994) Genetic analysis of RXRα developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. Cell 78:987–1003

Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, Decimo D, Krezel W, Dierich A, Chambon P (1996) Abnormal spermatogenesis in RXRβ mutant mice. Genes Dev 10:80–92

Kostrouch Z, Kostrouchova M, Love W, Jannini E, Piatigorsky J, Rall JE (1998) Retinoic acid X receptor in the diploblast, *Tripedalia cystophora*. Proc Natl Acad Sci USA 95:13442–13447

Krezel W, Dupe V, Mark M, Dierich A, Kastner P, Chambon P (1996) RXRγ null mice are apparently normal and compound RXRα$^{+/-}$/RXRβ$^{-/-}$/RXRγ$^{-/-}$ mutant mice are viable. Proc Natl Acad Sci USA 93:9010–9014

Laudet V (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. J Mol Endocrinol 19:207–226

LeBlanc GA, Campbell PM, den Besten P, Brown RP, Chang ES, Coats JR, de Fur PL, Dhadialla T, Edwards J, Riddiford LM, Simpson MG, Snell TW, Thorndyke M, Matsumura F (1999) The endocrinology of invertebrates. In: deFur P, Crane M, Ingersoll C, Tattersfield L (eds) Endocrine disruption in invertebrates: endocrinology, testing and assessment. SETAC Press, Pensacola, FL, pp 23–106

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA (1995) An antidiabetic thiazolidinedione is a high-affinity ligand for peroxisome proliferator-activated receptor gamma. J Biol Chem 270:12953–12956

Maglich JM, Sluder A, Guan X, Shi Y, Mckee DD, Carrick K, Kamdar K, Wilson TM, Moore JT (2001) Comparison of complete nuclear receptor sets from the human, *Caenorhabditis elegans* and *Drosophila* genomes. Genome Biol 2:1–7

Mangelsdorf DJ, Borgemeyer U, Heyman RA, Yang-Zhou J, Ong ES, Oro AE, Kakizuka A, Evans RM (1992) Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. Genes Dev 6:329–344

Mangelsdorf DJ, Evans RM (1995) The RXR heterodimers and orphan receptors. Cell 83:841–850

Matthiessen P, Reynoldson T, Billinghurst Z, Brassard DW, Cameron P, Chandler GT, Davies IM, Horiguchi T, Mount DR, Oehlmann J, Pottinger TG, Sibley PK, Thompson HM, Vethaak AD (1999) Field assessment for endocrine disruption in invertebrates. In: deFur P, Crane M, Ingersoll C, Tattersfield L (eds) Endocrine disruption in invertebrates: endocrinology, testing and assessment. SETAC Press, Pensacola, FL, pp 199–270

Mukherjee R, Davies PJA, Crombie DL, Bischoff ED, Cesario RM, Jow L, Hamann LG, Boehm MF, Mondon CE, Nadzan AM, Paterniti Jr JR, Heyman RA (1997) Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Nature 386:407–410

Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, Takatori S, Kitagawa Y, Hori S, Utsumi H (2000) Estrogenic activities of 517 chemicals by yeast two-hybrid assay. J Health Sci 46:282–298

Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T (2004) Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environ Sci Technol 38:6271–6276

Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfold MG, Willson TM, Glass CK, Milburn MV (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor γ. Nature 395:137–143

Oberdörster E, McClellan-Green P (2000) The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta*. Peptides 21:1323–1330

Oberdörster E, McClellan-Green P (2002) Mechanisms of imposex induction in the mud snail, *Ilyanassa obsoleta*: TBT as a neurotoxin and aromatase inhibitor. Mar Environ Res 54:715–718

Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H, Moras D (1995) Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. Nature 378:681–689

Ronis MJJ, Mason AZ (1996) The metabolism of testosterone by the periwinkle (*Littorina littorea*) in vitro and in vivo: effects of tributyltin. Mar Environ Res 42:161–166

Saitoh M, Yanase T, Morinaga H, Tanabe M, Mu YM, Nishi Y, Nomura M, Okabe T, Goto K, Takayanagi R, Nawata H (2001) Tributyltin or triphenyltin inhibits aromatase activity in human granulosa-like tumor cell line KGN. Biochem Biophys Res Commun 289:198–204

Smith BS (1971) Sexuality in the American mud snail, *Nassarius obsoletus* Say. Proc Malacol Soc Lond 39:377–378

Sohoni P, Sumpter JP (1998) Several environmental estrogens are also anti-androgens. J Endocrinol 158:327–339

Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR, Evans RM (1994) RXRα mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. Genes Dev 8:1007–1018

ten Hallers-Tjabbes CC, Kemp JF, Boon JP (1994) Imposex in whelks *Buccinum undatum* from the open North Sea: relation to shipping traffic intensities. Mar Pollut Bull 28:311–313

Terlizzi A, Delos AL, Garaventa F, Faimali M, Geraci S (2004) Limited effectiveness of marine protected areas: imposex in *Hexaplex trunculus* (Gastropoda, Muricidae) populations from Italian marine reserves. Mar Pollut Bull 48:188–192

Terlizzi A, Geraci S, Minganti V (1998) Tributyltin (TBT) pollution in the coastal waters of Italy as indicated by imposex in *Hexaplex trunculus* (Gastropoda, Muricidae). Mar Pollut Bull 36:749–752

Thornton JW, Need E, Crews D (2003) Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. Science 301:1714–1717

Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79:1147–1156

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

Tomohiko Kanayama, Naoki Kobayashi, Satoru Mamiya, Tsuyoshi Nakanishi, and Jun-ichi Nishikawa

Departments of Environmental Biochemistry (T.K., N.K., S.M., J.N.) and Toxicology (T.N.), Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

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-

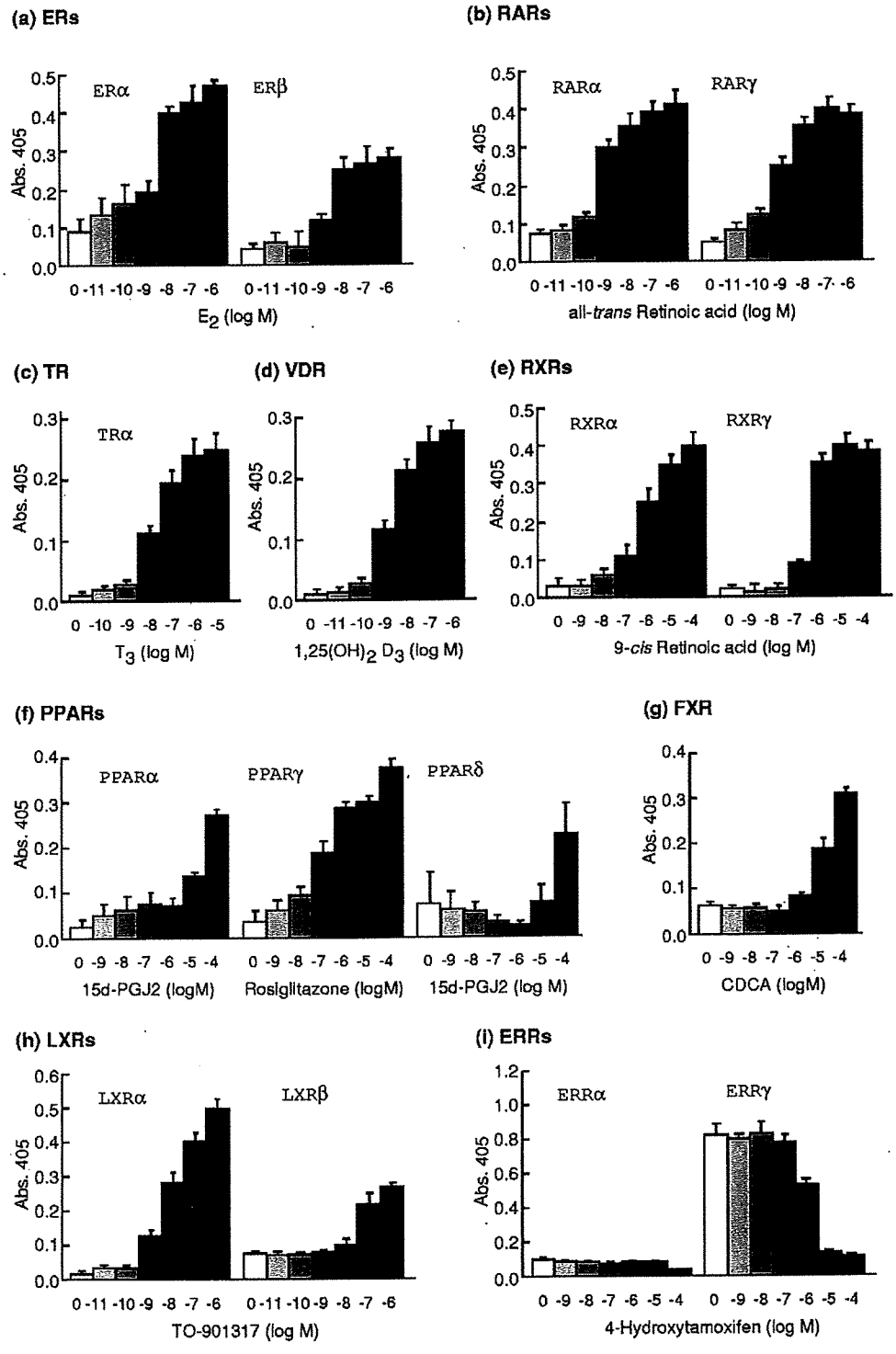

Fig. 1. Ligand-dependent interaction of nuclear receptor and TIF2 in vitro. Ligand-dependent interactions between nuclear receptors and TIF2-BAP were determined as relative alkaline phosphatase activity (vertical axis). The receptor-ligands pairs tested were ERα/β-17β-estradiol (E_2), RARα/γ-all-*trans*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

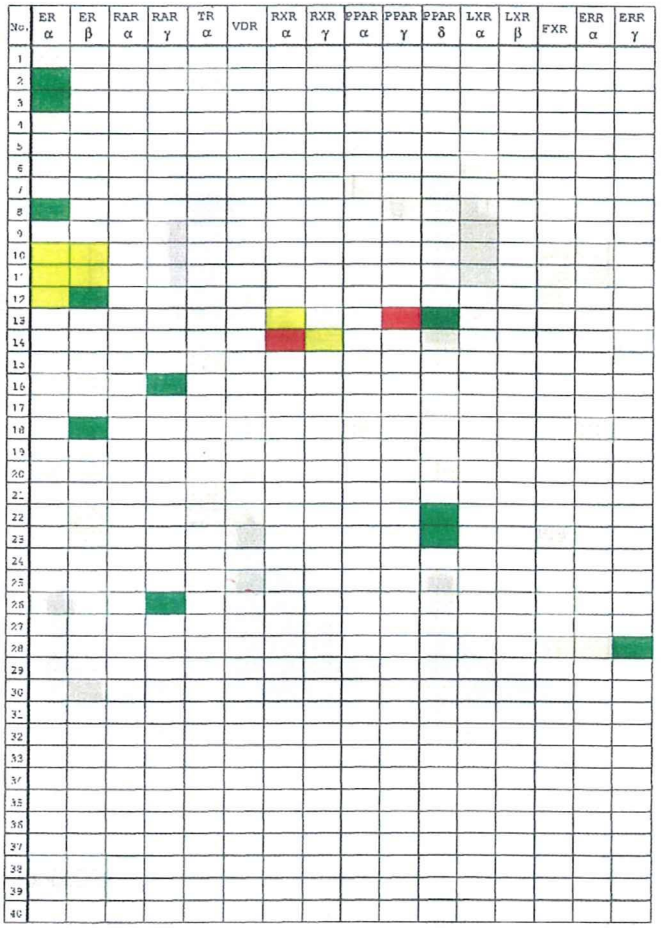

Fig. 2. Agonistic activities of suspected endocrine disruptors for various nuclear receptors. The effects of chemicals on the interaction between nuclear receptors and the coactivator TIF2 were assessed using the CoA-BAP system. The numbers in the far left column correspond to the chemicals listed in 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

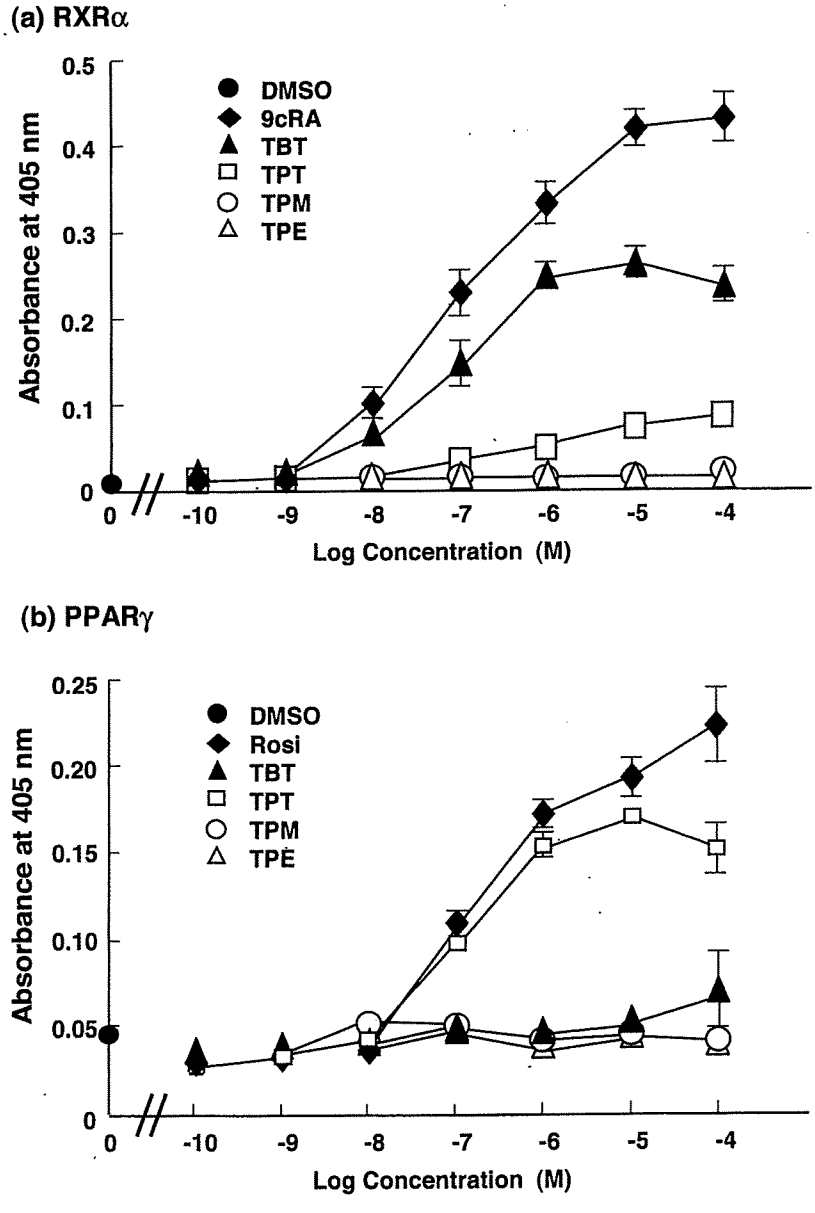

Fig. 3. Dose-response curves of the effects of organotin compounds on hRXRα and human PPARγ (hPPARγ) in the CoA-BAP system. A, TBT (▲) showed strong agonistic activity for hRXRα at as low a concentration as that of 9-*cis* retinoic acid (9cRA, ♦). B, TPT (□) showed strong agonistic activity to hPPARγ 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, 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.

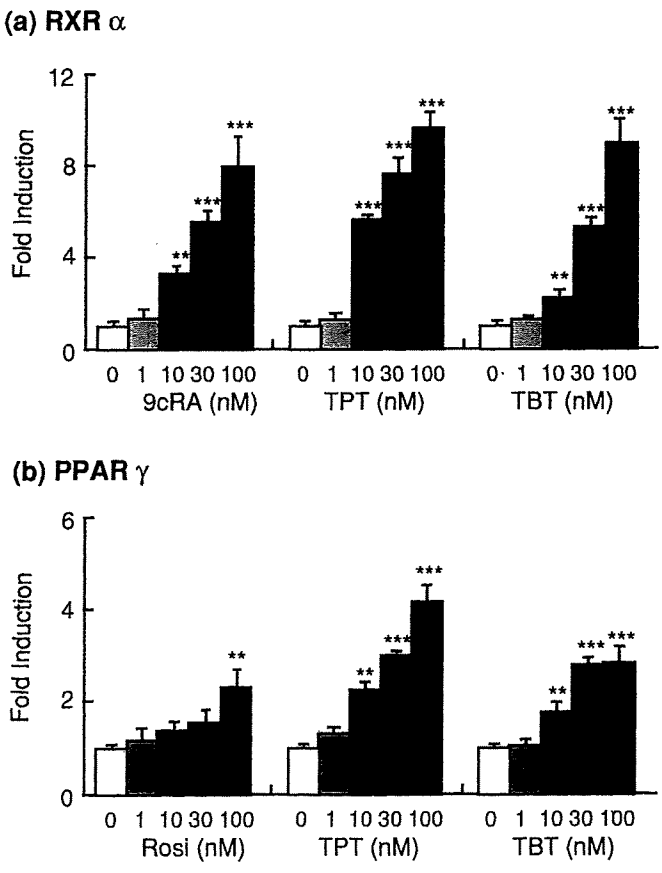

Fig. 4. Organotin compounds induce transcriptional activity through RXRα and PPARγ. Ligand-dependent transactivation of RXRα and PPARγ were detected as luciferase activity. a, F9 cells were cotransfected with a GAL4-DBD-hRXRα expression plasmid and a GAL4-responsive reporter plasmid. b, NIH-3T3 cells 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.

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/

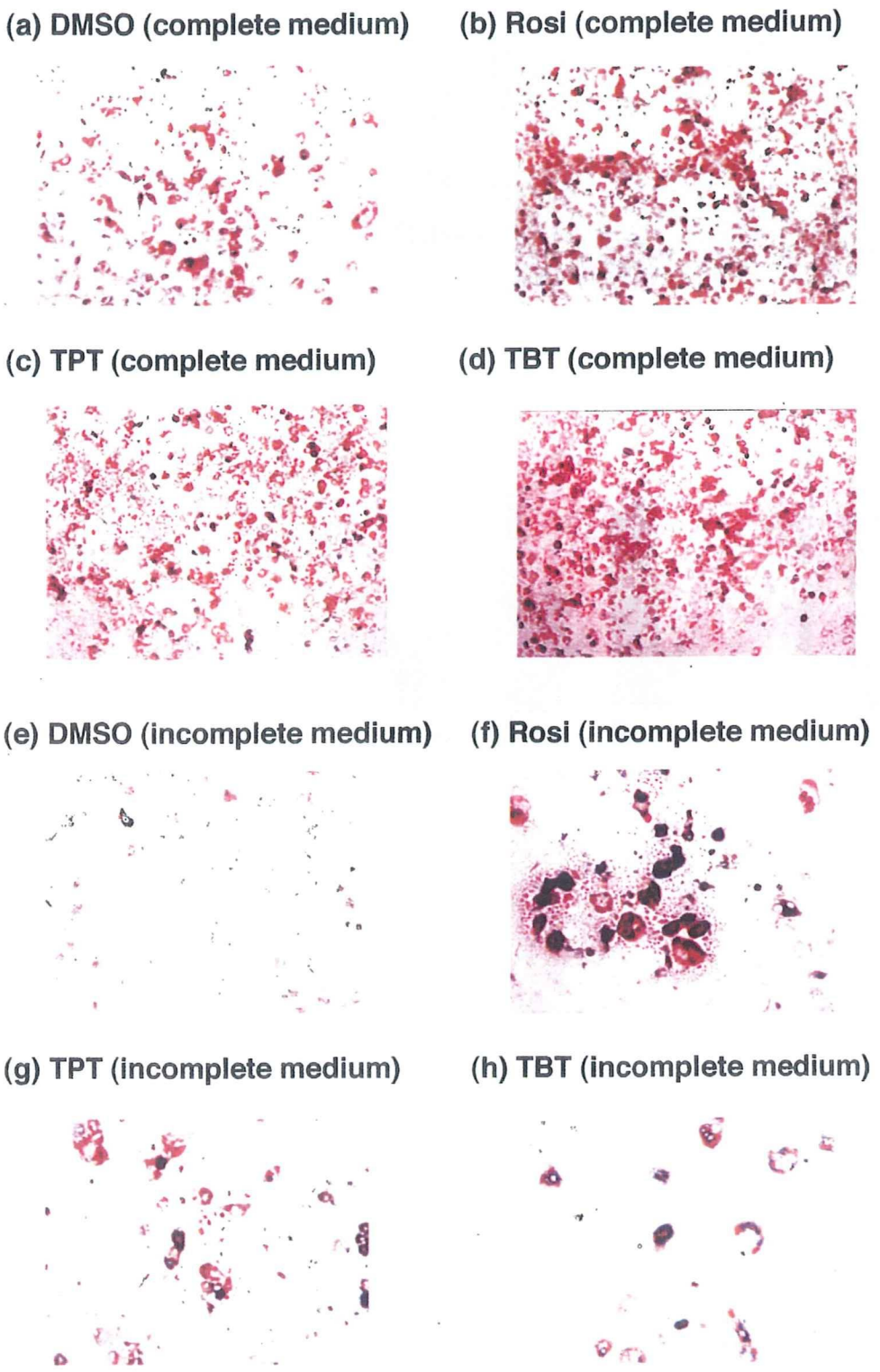

Fig. 5. Enhancement of lipid accumulation by organotin compounds. 3T3-L1 cells were maintained in DMEM containing 10% calf serum. One day after reaching confluence, the cells were treated for 60 h with vehicle only (a and e), 100 nM rosiglitazone (b and f), 100 nM TPT (c and g), or 100 nM TBT (d and 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.

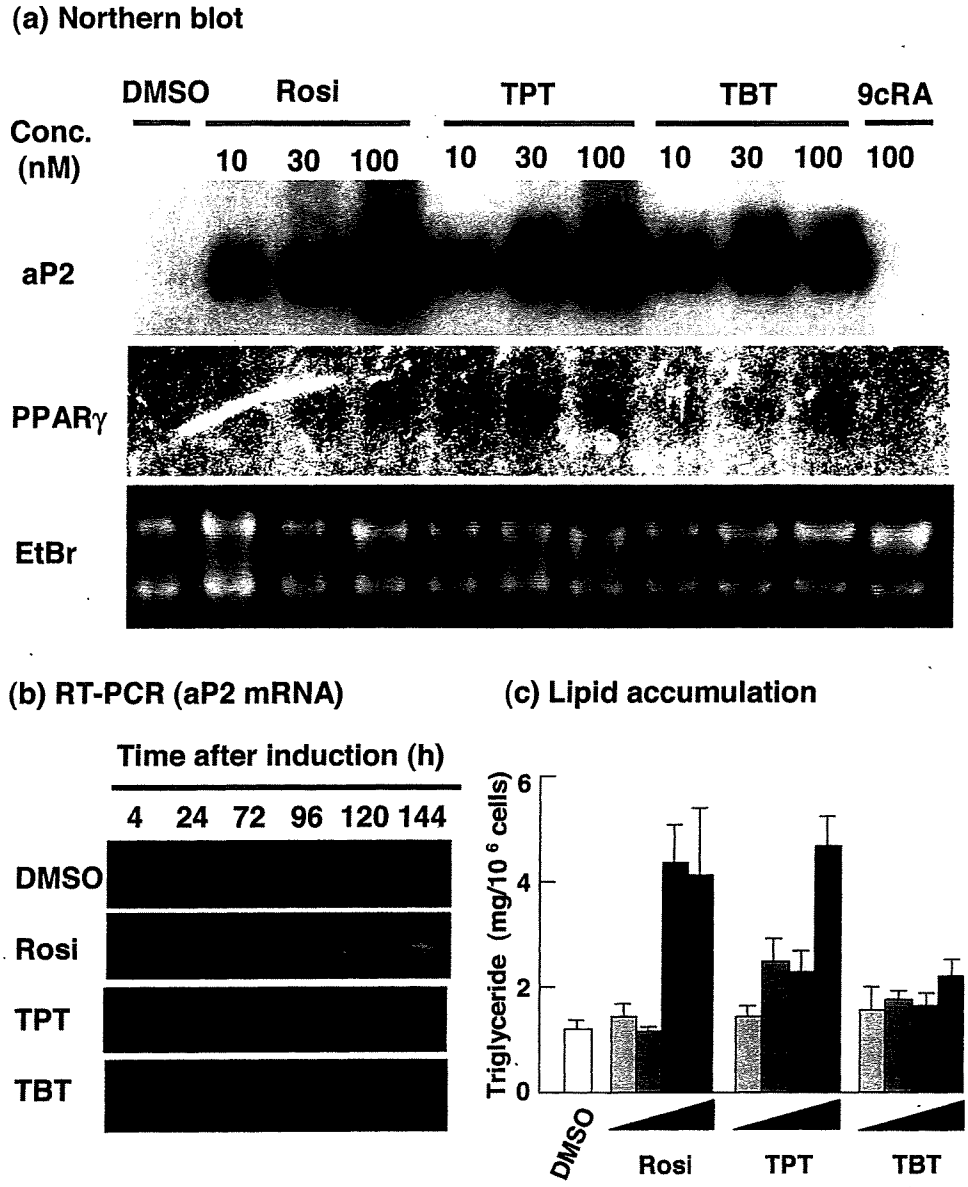

Fig. 6. Induction of adipocyte differentiation markers by organotin compounds. a, induction of adipocyte marker genes by organotin compounds in incomplete differentiation medium. 3T3-L1 cells were maintained in DMEM containing calf serum. One day after reaching confluence, the cells were treated with 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.

References

Adeeko A, Forsyth DS, Casey V, Cooke GM, Barthelemy, J Cyr DG, Trasler JM, Robair B, and Hales BF (2003) Effects of in utero tributyltin chloride in the rat on pregnancy outcome. *Toxicol Sci* **74**:407–415.

Bettin C, Oehlmann J, and Stroben E (1996) TBT-induced imposex in marine neo-gastropods is mediated by an increasing androgen level. *Helgol Meeresunters* **50**:299–317.

Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong, W Shi L, Perkins R, and Sheehan DM (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol Sci* **54**:138–153.

Boyer IJ (1989) Toxicity of dibutyltin, tributyltin and other organotin compounds to human and to experimental animals. *Toxicology* **55**:253–298.

Bryan GW, Gibbs PE, Hummerstone LG, and Burt GR (1986) The decline of the gastropod *Nucella lapillus* around south-west England: evidence for the effect of tributyltin from anti-fouling paints. *J Mar Biol Assoc UK* **66**:611–640.

Chawla A, Repa JJ, Evans RM, and Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science (Wash DC)* **294**:1866–1870.

Cooke GM (2002) Effect of organotins on human aromatase activity in vitro. *Toxicol Lett* **126**:121–130.

Coward P, Lee D, Hull MV, and Lehmann JM (2001) 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor gamma. *Proc Natl Acad Sci USA* **98**:8880–8884.

Duvic M, Hymes K, Heald P, Breneman D, Martin AG, Myskowski P, Crowley C, and Yocum RC (2001) Bexarotene is effective and safe for treatment of refractory advanced-stage cutaneous T-cell lymphoma: multinational phase II-III trial results. *J Clin Oncol* **19**:2456–2471.

Escriva H, Safi R, Hanni C, Langlois MC, Saumitou-Laprade P, Stehelin D, Capron A, Pierce R, and Laudet V (1997) Lignad binding was acquired during evolution of nuclear receptors. *Proc Natl Acad Sci USA* **94**:6803–6808.

Fent K (1996) Ecotoxicology of organotin compounds. *Crit Rev Toxicol* **26**:1–117.

Gibbs PE and Bryan GW (1986) Reproductive failure in populations of the dog-whelk, *Nucella lapillus*, caused by imposex induced by tricutyltin from antifouling paints. *J Mar Biol Assoc UK* **66**:767–777.

Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, et al. (2001) Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update* **7**:248–264.

Heidrich DD, Steckelbroek S, and Klingmuller D (2001) Inhibition of human cytochrome P450 aromatase activity by butyltins. *Steroids* **66**:763–769.
Horiguchi T, Shiraishi H, Shimizu M, and Morita M (1997) Effects of triphenytin chloride and five other organotin compounds on the development of imposex in the rock shell, *Thais clavigera*. *Pollution* **95**:85–91.
Kamei Y, Ohizumi H, Fujitani Y, Nemoto T, Tanaka T, Takahashi N, Kawada T, Miyoshi M, Ezaki O, and Kakizuka A (2003) PPARγ coactivator 1β/ERR ligand 1 is an ERR protein ligand, whose expression induces a high-energy expenditure and antagonizes obesity. *Proc Natl Acad Sci USA* **100**:12378–12383.
Kanayama T, Mamiya S, Nishihara T, and Nishikawa J (2003) Basis of a high-throughput method for nuclear receptor ligands. *J Biochem* **133**:791–797.
Kodera Y, Takeyama K, Murayama A, Suzawa M, Masuhiro Y, and Kato S (2000) Ligand type-specific interactions of perioxisome proliferator-activated receptor γ with transcriptional coactivators. *J Biol Chem* **275**:33201–33204.
Laudet V (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J Mol Endocrinol* **19**:207–226.
Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, and Kliewer SA (1995) An antidiabetic thiazolidinedione is a high-affinity ligand for peroxisome proliferator-activated receptor γ. *J Biol Chem* **270**:12953–12956.
Liu S, Ogilvie KM, Klausing K, Lawson MA, Jolley D, Li D, Bilakovics J, Pascual B, Hein N, Urcan M, et al. (2002) Mechanism of selective retinoid X receptor agonist induced hypothyroidism in the rat. *Endocrinology* **143**:2880–2885.
Mangelsdorf DJ and Evans RM (1995) The RXR heterodimers and orphan receptors. *Cell* **83**:841–850.
Matthiessen P and Gibbs PE (1998) Critical appraisal of the evidence for tributyltin mediated endocrine disruption in mollusks. *Environ Toxicol Chem* **17**:37–43.
Morcillo Y and Porte C (1999) Evidence of endocrine disruption in the imposex-affected gastropod *Bolinus brandaris*. *Environ Res* **81**:349–354.
Mukherjee R, Davies PJA, Crombie DL, Bischoff ED, Cesario RM, Jow L, Hamann LG, Boehm MF, Mondon CE, Nadzan AM, et al. (1997) Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature (Lond)* **386**:407–410.
Nakanishi T, Kohroki J, Suzuki S, Ishizaki J, Hiromori Y, Takasuga S, Itoh N, Watanabe Y, Utoguchi N, and Tanaka K (2002) Trialkyltin compounds enhance human CG secretion and aromatase activity in human placental choriocarcinoma cells. *J Clin Endocrinol Metab* **87**:2830–2837.
Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, Takatori S, Kitagawa Y, Hori S, and Utsumi H (2000) Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J Health Sci* **46**:282–298.
Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, and Horiguchi T (2004) Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropod. *Environ Sci Technol* **38**:6271–6276.
Piver WT (1973) Organotin compounds: industrial applications and biological investigation. *Environ Health Pespect* **4**:61–79.
Saitoh M, Yanase T, Morinaga H, Tanabe M, Mu YM, Nishi Y, Nomura M, Okabe T, Goto K, Takayanagi R, et al. (2001) Tributyltin or triphenytin inhibits aromatase activity in human granulosa-like tumor cell line KGN. *Biochem Biophys Res Commun* **289**:198–204.
Sanderson JT, Boerma J, Lansbergen GW, and van den Berg M (2002) Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicol Appl Pharmacol* **182**:44–54.
Smith BS (1971) Sexuality in the American mud snail, *Nassarius obsoletus* Say. *Proc Malac Soc Lond* **39**:377–378.
Smith CL and O'Malley BW (2004) Coregulator function: a key to understanding tissue specificity of receptor modulators. *Endocr Rev* **25**:45–71.
Sohoni P and Sumpter JP (1998) Several environmental estrogens are also anti-androgens. *J Endocrinol* **158**:327–339.
Sultan C, Balaguer P, Terouanne B, Geoget V, Paris F, Jeandel C, Lumbroso S, and Nicolas J (2001) Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. *Mol Cell Endocrinol* **10**:99–105.
Tontonoz P, Hu E, and Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* **79**:1147–1156.
Tremblay GB, Kunath T, Bergeron D, Lapointe L, Champigny C, Bader JA, Rossant J, and Giguere V (2001) Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear ERRβ. *Genes Dev* **15**:833–838.
Yaki-Jarvinen H (2004) Thiazolidinediones. *N Engl J Med* **351**:1106–1118.

Address correspondence to: Dr. Jun-ichi Nishikawa, Department of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: nisikawa@phs.osaka-u.ac.jp

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Toxicology Letters 155 (2005) 329–335
Toxicology Letters
www.elsevier.com/locate/toxlet

Some organotin compounds enhance histone acetyltransferase activity

Shigehiro Osada[a,*], Jun-ichi Nishikawa[a], Tsuyoshi Nakanishi[b], Keiichi Tanaka[b], Tsutomu Nishihara[a]

[a] *Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-Oka, Suita, Osaka 565-0871, Japan*
[b] *Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-Oka, Suita, Osaka 565-0871, Japan*

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-

* Corresponding author. Tel.: +81 6 6879 8242; fax: +81 6 6879 8244.
E-mail address: osada@phs.osaka-u.ac.jp (S. Osada).

0378-4274/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.toxlet.2004.10.009

sists of two each of the core histones H2A, H2B, H3, and H4, all of which have a basic, unstructured amino terminal tail. These basic proteins are susceptible to a variety of posttranslational modifications, e.g., acetylation (Howe et al., 1999; Wu et al., 1986). One of the well-characterized modifications is acetylation of specific lysine residues, which is reversibly catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC).

The GCN5-related *N*-acetyltransferase family includes GCN5 and PCAF, which share a remarkable degree of homology throughout their sequences and are present in a multisubunit complex consisting of more than 20 distinct polypeptides (Ogryzko et al., 1998). GCN5 and PCAF are transcriptional coactivators with intrinsic HAT activity; they contribute to transcriptional activation by acetylating chromatin (Sterner and Berger, 2000). Disruption of *Gcn5* and *Pcaf* genes revealed that they play distinct but functionally overlapping roles during embryogenesis (Yamauchi et al., 2000). Other well-characterized coactivators possessing HAT activity are CBP and P300, which are ubiquitously expressed global transcriptional coactivators that have critical roles in a wide variety of cellular processes, including development (Giles et al., 1998; Giordano and Avantaggiati, 1999; Yao et al., 1998).

Recent studies have demonstrated that some environmental pollutants affect the hormonal system and produce adverse effects on animals and probably also humans (Colborn et al., 1996; Van der Kraak et al., 1992). These pollutants are referred to as endocrine-disrupting chemicals (EDCs). The major targets of EDCs are nuclear hormone receptors, which bind steroid hormones and regulate transcription of their target genes (Nishihara et al., 2000; Nishikawa et al., 1999). For ligand-dependent gene activation, nuclear hormone receptors require coactivators that link the basal transcriptional machinery with the hormone receptors (Chen, 2000). Recent studies have shown that the nuclear hormone receptor coactivators possess HAT activity and recruit two other types of HATs, CBP and PCAF (Chen et al., 1997; Spencer et al., 1997). Hormone-dependent gene activation mediated by nuclear receptors involves the mutual recruitment of at least three classes of HATs.

These observations raise the possibility that HATs may be the targets of EDCs, and we tested the effects of suspected EDCs on HAT activity. Interestingly tributyltin (TBT) and triphenyltin (TPT) enhanced HAT activity, but other EDCs did not. These organotin chemicals have been used in such applications as wood preservation and as antifouling agents in marine paints, and are ubiquitous in the environment. TBT and TPT have been found to induce imposex (the superimposition of male sex organs in female gastropods) in the rock shell *Thais clavigera* and are known EDCs in marine species (Horiguchi et al., 1997). These compounds are also reported to affect not only the hormone system but also embryogenesis in mammals (Harazono et al., 1998; Nakanishi et al., 2002). Organotins caused behavioral and neurological symptoms and pancreatic and hepatic toxicities in rodents (Brown et al., 1979; Merkord et al., 2001). In the immune system, at low doses TBT inhibits immature thymocyte proliferation, whereas at higher doses in particular TBT induces apoptotic cell death (Gennari et al., 2002). However, the biological mechanism of the effects of organotin compounds on marine species and mammals awaits further characterization. The present study showed that some organotin compounds enhanced HAT activity when both core and nucleosomal histones were used as substrates. These data suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

2. Materials and methods

2.1. Chemicals

All chemicals were dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemicals, Osaka, Japan). Organotin and related chemicals tested are listed in Table 1.

2.2. Preparation of rat liver nuclear extracts and HAT fraction

All animal care and handling procedures were approved by the animal care and use committee of Osaka University. Rat liver nuclear extracts (RLNE) were prepared as described previously (Osada et al., 1995). For binding RLNE to Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany), nuclei were suspended in a nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and

Table 1
Effect of organotin and related chemicals on HAT activity

Chemical	Abbreviation	Relative HAT activity[a]	CAS no.	Purity (%)	Source
Triphenyltin chloride	TPT	$2.03 \pm 0.13^{**}$	639-58-7	>95	Aldrich Chemicals
Diphenyltin dichloride	DPT	$1.63 \pm 0.061^{**}$	1135-99-5	>96	Aldrich Chemicals
Monohenyltin trichloride	MPT	0.97 ± 0.026	1124-19-2	>98	Aldrich Chemicals
Tributyltin chloride	TBT	$2.22 \pm 0.13^{**}$	1461-22-9	>95	Tokyo Kasei Kogyo
Dibutyltin dichloride	DBT	$1.81 \pm 0.045^{**}$	683-18-1	>97	Tokyo Kasei Kogyo
Monobutyltin trichloride	MBT	1.01 ± 0.037	1118-46-3	>95	Aldrich Chemicals
Tin chloride	$SnCl_4$	0.91 ± 0.025	10025-69-1	>97	Nacalai tesque
Tetrabutyltin	TetBT	1.05 ± 0.042	1461-25-2	>93	Aldrich Chemicals
Trimethyltin chloride	TMT	0.95 ± 0.011	1066-45-1	>98	Aldrich Chemicals
Triethyltin bromide	TET	$1.27 \pm 0.034^{*}$	2767-54-6	>97	Aldrich Chemicals
Tripropyltin chloride	TPrT	$3.09 \pm 0.080^{**}$	2279-76-7	>98	Merck Schuchardt
Triphenylsilanol	TPSiOH	1.14 ± 0.23	791-31-1	>95	Merck Schuchardt
Triphenylmethane	TPM	0.95 ± 0.12	519-73-3	>98	Kanto Chemical
Triphenylethylene	TPE	0.91 ± 0.12	58-72-0	>98	Kanto Chemical

[a] Core histones and 10 μM of chemicals were used for the HAT assay. Relative HAT activity shows mean HAT activities (±S.D.) relative to a control treated without chemical (three independent experiments). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)), and 3 M KCl was added to a final concentration of 0.55 M. Nuclear lysate was gently mixed on ice for 30 min and centrifuged at 40,000 rpm in a Beckman 50.2 Ti rotor (Beckman, Fullerton, CA, USA) for 40 min at 4 °C. The RLNE was diluted by adding the same volume of nuclear lysis buffer without KCl and $MgCl_2$ and then incubating with preequilibrated Ni^{2+}-NTA agarose overnight at 4 °C on a rotating wheel. This suspension was poured into a column and the flowthrough was collected. The column was then washed with five column volumes of the nuclear lysis buffer without KCl and $MgCl_2$, and the retained proteins were eluted with a solution consisting of 20 mM imidazole (pH 7.5), 100 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 1% protease inhibitor cocktail.

2.3. Western blotting and antibodies

Ten microliters of each fraction of column eluate was electrophoresed on an SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with an ECL Western blotting analysis detection system (Amersham Biosciences, Piscataway, NJ, USA). Antibodies against P300, CBP, and GCN5 were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-PCAF antibody was kindly provided by Dr. Yoshihiro Nakatani (Harvard Medical School, Boston, MA, USA).

2.4. HAT assay

HAT assays were performed as follows: 1.5 μg core histones or nucleosome histones was incubated together with 5 μl of eluate from the Ni^{2+}-NTA agarose column, ^{3}H-labeled acetyl-CoA (0.25 μCi), and 1.2 μl of the test compound in 30 μl of HAT buffer (50 mM Tris–HCl, pH 8.0, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate) at 30 °C for 30 min. Histones were prepared as described previously (Owen-Hughes et al., 1999). After incubation, 15 μl of reaction mixture was transferred to a P81 phosphocellulose filter (Whatman, Brentford, UK) and allowed to air-dry. Filters were washed three times in wash buffer (50 mM $NaHCO_3$-$NaCO_3$, pH 9.2) and air-dried. The samples were counted in a scintillation counter (Beckman) for 10 min. The remaining 15 μl was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). All gels were stained with Coomassie Brilliant blue to ensure loading of equivalent amounts of histone in each lane, then destained and flourographed.

2.5. Statistics

All results are expressed as means ± standard deviations (S.D.). Statistical analysis was performed by Dunnett's method.

3. Results

3.1. Partial purification of HAT complex from RLNE

To observe the effects of suspected EDCs on HAT activity, we used partly purified HAT complex from RLNE. Many HATs function as catalytic subunits in HAT complexes, and the specificity and the activity of HAT complexes are different from those of recombinant HATs. For example, recombinant GCN5 can acetylate core histones well, but it exhibits poor nucleosomal HAT activity (Balasubramanian et al., 2002; Grant et al., 1997). Recombinant SAS2 does not show HAT activity, but a complex including SAS2 can acetylate histones (Sutton et al., 2003). Grant and coworkers reported that some native HAT complexes in yeast bind to Ni^{2+}-NTA agarose (Grant et al., 1997). We wondered whether native mammalian HAT complexes bind to Ni^{2+}-NTA agarose. RLNE was incubated with Ni^{2+}-NTA agarose, and bound proteins were eluted with a buffer containing imidazole. Bound fraction included at least four HATs: GCN5, PCAF, P300, and CBP (Fig. 1).

3.2. Effect of TBT and TPT on core HAT activity

Nickel and curcumin (diferuloylmethane) were found to inhibit the acetylation of histones in vitro using recombinant histone acetyltransferase (Balasubramanyam et al., 2004; Broday et al., 2000), indicating that heavy metals and low molecular compounds may affect HAT activity. Suspected EDCs were screened for inhibition and activation of the HAT activity of partly purified HATs by the standard HAT assay procedure using core histone as substrate (Eberharter et al., 1998) and 20 chemicals in the list of chemicals suspected of having endocrine disrupting effects, as published by the Strategic Programs on Environmental Endocrine Disrupters '98, the Japan Environmental Agency (www.env.go.jp/en/pol/speed98/sp98.pdf). Tested 20 chemicals (benzophenone, octachlorostyrene, diethyl phthalate, butyl benzyl phthalate, diethylhexyl adipate, TPT, diethylhexyl phthalate, dicyclohexyl phthalate, di-*n*-butyl phthalate, TBT, 4-nonylphenol, *p*-octylphenol, bisphenol A, 2,4-dichlorophenol, 4-nitrotoluene, di-*n*-pentyl phthalate, dipropyl phthalate, pentachlorophenol, amitrole, and dihexyl phthalate) did not inhibit HAT activity. Interestingly TBT and TPT enhanced HAT activity but other chemicals did not (Fig. 2A and data not shown). HAT activity in the presence of various concentrations of TBT and TPT was assayed, and both chemicals demonstrated dose-dependent enhancement of HAT activity (Fig. 2A). TBT and TPT at both 1 and 10 μM promoted HAT activity but at 0.1 μM had little, if any, effect. Fluorography indicated that partly purified HATs from RLNE acetylated histones H2A, H3 and H4 and that these histones were more effectively acetylated in the presence of TBT or TPT (Fig. 2A, middle panel). A partly purified HAT fraction includes several kinds of HATs and HDACs, but TBT and TPT did not show HDAC inhibitory activity (data not shown). HDACs are classified into three groups, class I, II, and III. Assay reaction mixtures include butyrate, an inhibitor for class I and II HDACs (Ajamian et al., 2004). Further, TBT and TPT enhanced HAT activity in the presence of nicotinamide, an inhibitor for class III HDACs (Bitterman et al., 2002) (data not shown). These results also indicate that TBT and TPT do not inhibit HDAC activity.

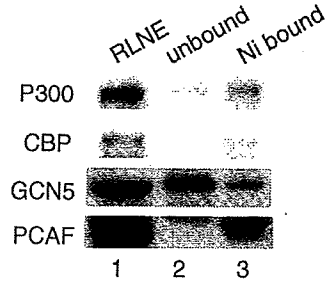

Fig. 1. Partial purification of HATs from RLNE. Western blots were performed with RLNE (lane 1), flow-through from a Ni^{2+}-NTA agarose column (unbound fraction, lane 2), and eluate from a Ni^{2+}-NTA agarose column (Ni-bound, lane 3) using antibodies against P300, CBP, GCN5, and PCAF.

We investigated the effects of organotin and related chemicals on HAT activity. TBT is metabolized to dibutyltin (DBT), monobutyltin (MBT), and inorganic tin; and TPT is metabolized to diphenyltin (DPT), monophenyltin (MPT), and inorganic tin (Horiguchi et al., 1997). The effects of these metabolites of the organotin compounds on HAT activity were also analyzed (Table 1). DBT and DPT enhanced HAT activity, but MBT, MPT, and $SnCl_4$ had no effect. DBT and DPT showed less enhancement of HAT activity than TPT and TBT, but tetrabutyltin did not affect HAT activity (Table 1). These results indicate that trialkyltin com-

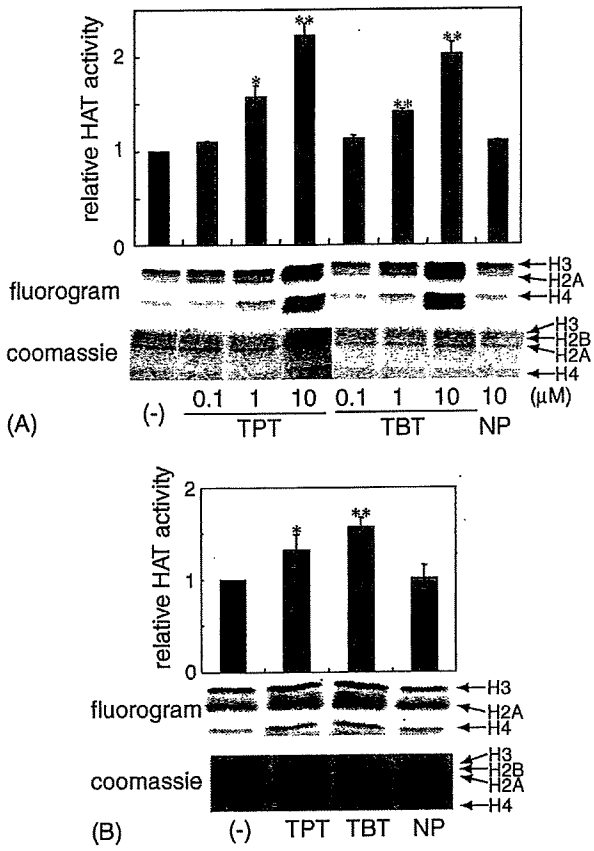

Fig. 2. Effect of TPT and TBT on HAT activity. (A) HAT assays with core histones as substrates and partly purified HAT fraction from RLNE as enzyme. The bar graph shows mean HAT activities (±S.D.) relative to a control treated without chemical (three independent experiments). 4-nonylphenol (NP) was used as a negative control. $^{*}P<0.05$ and $^{**}P<0.01$ compared to control. Products of HAT assays were separated by SDS-PAGE on 18% polyacrylamide gels to resolve the histones. The gels were stained with Coomassie Brilliant blue to visualize proteins (lower panel) and were then dried and visualized by fluorography (middle panel). (B) HAT assays in the absence or presence of chemicals (10 μM), TPT, TBT, and NP, using nucleosome histones as substrates were performed in triplicate as described in the legend to (A). A typical fluorogram from three independent experiments that showed similar results is shown.

pounds are more effective enhancers than other alkyltin compounds.

Next, we focused on the number of carbon alkyl chain in the trialkyltin compounds. Trimethyltin (TMT) did not affect HAT activity, and triethyltin (TET) was a poor promoter of HAT activity. Interestingly, tripropyltin was a more effective enhancer than the compounds containing one or more butyl or phenyl groups. The compounds containing carbon or silicon in place of tin were used to determine whether tin is essential for the promotion of HAT activity by organotin compounds. However, triphenylsilanol, triphenylmethane, and triphenylethylene did not affect HAT activity (Table 1).

3.3. Effect of TBT and TPT on nucleosomal HAT activity

Several transcription co-activators possess HAT activity, and the acetylation of nucleosomes associated with the promoter is correlated with transcriptional activation (Ikeda et al., 1999; Sterner and Berger, 2000; Utley et al., 1998). To investigate the effect of TPT and TBT on the nucleosomal HAT activity, we used nucleosomal histones as substrates instead of core histones (Fig. 2B). The partly purified HAT fraction from RLNE includes HAT activity for nucleosome histones, which was promoted by adding TPT and TBT to the reaction. This means that these compounds enhance the core HAT activity as well as the nucleosomal HAT activity.

4. Discussion

We demonstrated that HAT activity is enhanced by certain organotin compounds, including TPT and TBT. Here we found that: (1) trialkyltin compounds are more effective enhancers of HAT activity than mono- and dialkyltin compounds; (2) tin compounds with short alkyl chains showed no effect; and (3) the tin atom is important for the enhancement of HAT activity. On the basis of these results, it is likely that the acetyl CoA binding pocket (active site) or the substrate-binding site of HATs can tolerate a small compound, such as TMT or TET. These results suggest that both a carbon chain of a suitable length and a charge of tin are required for the regulating HAT activity. The crystal structure of HATs with acetyl CoA and/or histone has been determined (Dutnall et al., 1998; Rojas et al., 1999; Yan et al., 2000). Therefore, this information might be useful for analyzing the molecular mechanism of the enhancement of HAT activity by organotin compounds. However, we cannot rule out an alternative possibility that the organotin compounds affects histones. For example, organotin compounds may release histones from a inhibitor of acetyltransferase complex, which binds to histones and masks them from being HAT substrates

(Seo et al., 2001), or change the structure of the histone tails and making them better substrates. Zoroddu et al. propose that the binding of Ni (II) can produce a secondary structure with organized side-chain orientation in the amino terminal tail of histone H4 (Zoroddu et al., 2002). Some compounds including heavy metal(s) may affect histones. We used a partly purified HAT fraction so that the HAT complexes would be in their native form. To clarify the mechanism of HAT activity enhancement by organotin compounds, we are proceeding with identification of the specific HAT whose activity is activated by organotin compounds.

A cDNA microarray analysis revealed that expression of about 130 genes was induced by treatment of the ascidian *Ciona intestinalis* with TBT (Azumi et al., 2004). We previously reported that the level of the mRNA for aromatase/CYP19, which is essential for converting androgenic to estrogenic steroids, was increased by treatment of human choriocarcinoma JAR cells with TBT (Nakanishi et al., 2002). The mechanism of the induction of these mRNAs by TBT has not been elucidated yet. Aberrant HAT activity induced by TBT treatment might cause unusual expression of these genes.

HAT activity is required for the regulation of gene expression and histone acetylation has a fundamental biological role. Organotin compounds have various influences on physical function including the hormone and immune systems, embryogenesis, development, etc. In previous studies, reasonable butyltin concentrations were detected in human liver and blood (Kannan et al., 1999; Lo et al., 2003). Aberrant HAT activity in vivo induced by organotin compounds may cause abnormal development in human and wildlife. Our 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.

Acknowledgements

This research was supported in part by grants from the Japanese Ministry of Education, Science, Sports, and Culture and from the Takeda Science Foundation. We are grateful to Dr. Yoshihiro Nakatani (Harvard Medical School) for kindly providing anti-PCAF antibody. We also thank the staff of the Radioisotope Research Center, Osaka University.

References

Ajamian, F., Salminen, A., Reeben, M., 2004. Selective regulation of class I and class II histone deacetylases expression by inhibitors of histone deacetylases in cultured mouse neural cells. Neurosci. Lett. 365, 64–68.

Azumi, K., Fujie, M., Usami, T., Miki, Y., Satoh, N., 2004. A cDNA microarray technique applied for analysis of global gene expression profiles in tributyltin-exposed ascidians. Mar. Environ. Res. 58, 543–546.

Balasubramanian, R., Pray-Grant, M.G., Selleck, W., Grant, P.A., Tan, S., 2002. Role of the Ada2 and Ada3 transcriptional coactivators in histone acetylation. J. Biol. Chem. 277, 7989–7995.

Balasubramanyam, K., Varier, R.A., Altaf, M., Swaminathan, V., Siddappa, N.B., Ranga, U. and Kundu, T.K., 2004. Curcumin, a novel p300/CBP specific inhibitor of acetyltransferase, represses the acetylation of histones/nonhistone proteins and HAT dependent chromatin transcription. J. Biol. Chem. in press.

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., Sinclair, D.A., 2002. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J. Biol. Chem. 277, 45099–45107.

Broday, L., Peng, W., Kuo, M.H., Salnikow, K., Zoroddu, M., Costa, M., 2000. Nickel compounds are novel inhibitors of histone H4 acetylation. Cancer Res. 60, 238–241.

Brown, A.W., Aldridge, W.N., Street, B.W., Verschoyle, R.D., 1979. The behavioral and neuropathologic sequelae of intoxication by trimethyltin compounds in the rat. Am. J. Pathol. 97, 59–82.

Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y., Evans, R.M., 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90, 569–580.

Chen, J.D., 2000. Steroid/nuclear receptor coactivators. Vitam. Horm. 58, 391–448.

Colborn, T., Dumanoski, D., Myers, J.P., 1996. Our Stolen Future. Dutton, New York.

Dutnall, R.N., Tafrov, S.T., Sternglanz, R., Ramakrishnan, V., 1998. Structure of the histone acetyltransferase Hat1: a paradigm for the GCN5-related N-acetyltransferase superfamily. Cell 94, 427–438.

Eberharter, A., John, S., Grant, P.A., Utley, R.T., Workman, J.L., 1998. Identification and analysis of yeast nucleosomal histone acetyltransferase complexes. Methods 15, 315–321.

Gennari, A., Bol, M., Seinen, W., Penninks, A., Pieters, R., 2002. Organotin-induced apoptosis occurs in small CD4(+)CD8(+) thymocytes and is accompanied by an increase in RNA synthesis. Toxicology 175, 191–200.

Giles, R.H., Peters, D.J., Breuning, M.H., 1998. Conjunction dysfunction: CBP/p300 in human disease. Trends Genet 14, 178–183.