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

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ABSTRACT

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

TABLE 1
Suspected endocrine disruptors tested in this study

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

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

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

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

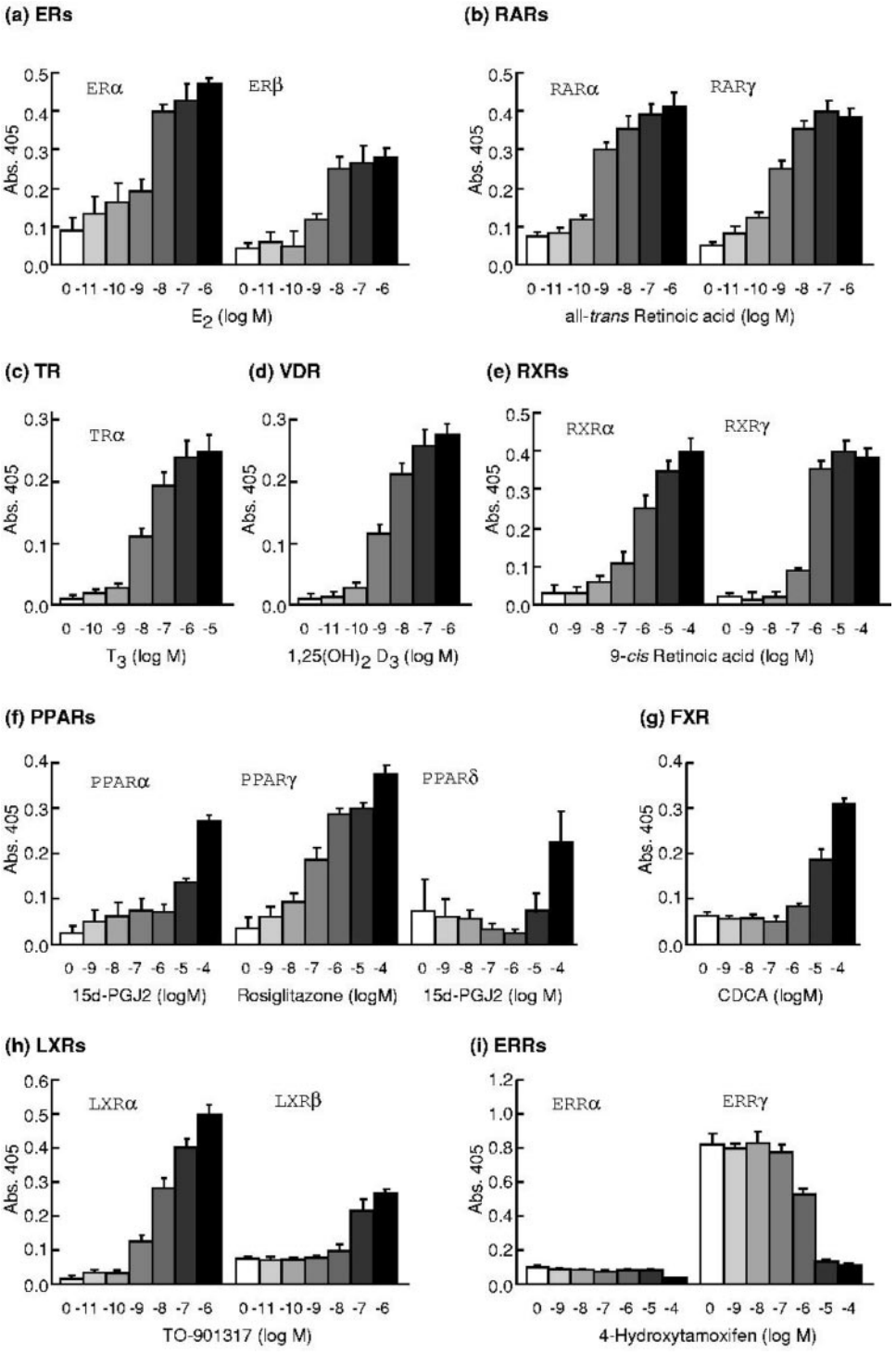

Fig. 1. Ligand-dependent interaction of nuclear receptor and TIF2 in vitro. Ligand-dependent interactions between nuclear receptors and TIF2-BAP were determined as relative alkaline phosphatase activity (vertical axis). The receptor-ligands pairs tested were ERα/β-17β-estradiol (E_2), RARα/γ-all-*trans* retinoic acid, TRα-3,5,3′-triiodo-L-thyronine (T_3), VDR-1α, 25-dihydroxy cholecalciferol [$1,25(OH)_2D_3$], RXRα/γ-9-*cis* retinoic acid, PPARα/δ-15-deoxy-^{12,14}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-

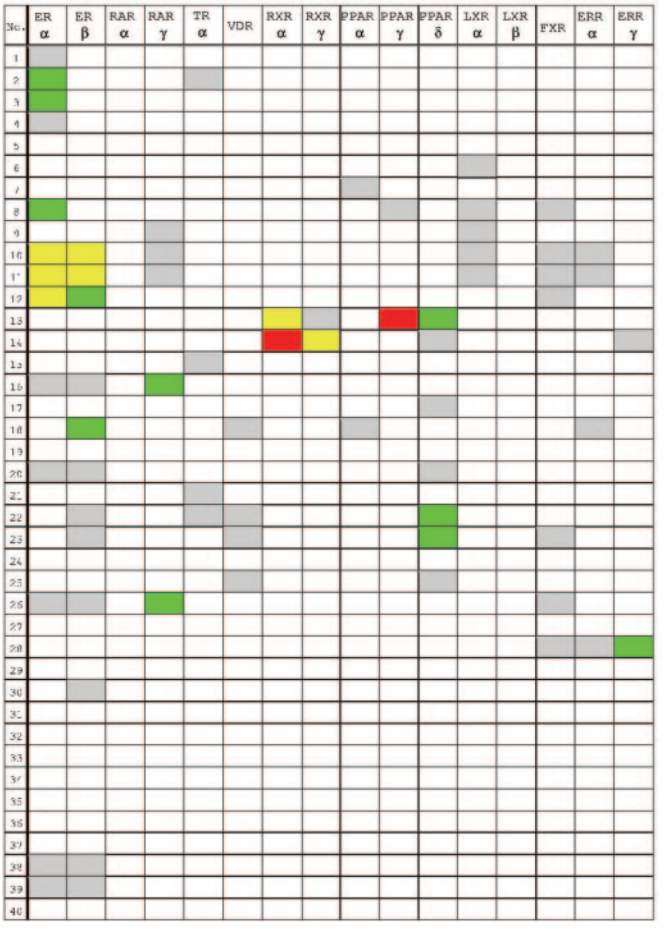

Fig. 2. Agonistic activities of suspected endocrine disruptors for various nuclear receptors. The effects of chemicals on the interaction between nuclear receptors and the coactivator TIF2 were assessed using the CoA-BAP system. The numbers in the far left column correspond to the chemicals listed in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

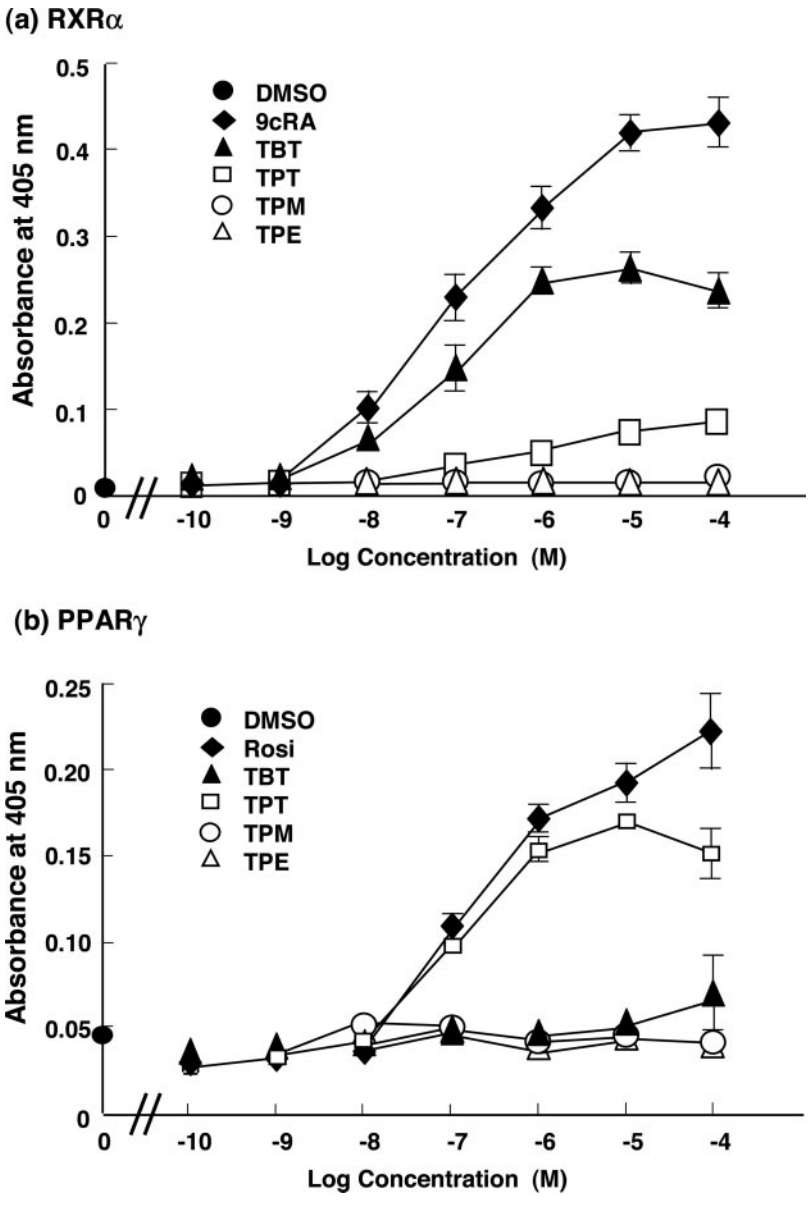

Fig. 3. Dose-response curves of the effects of organotin compounds on hRXRα and human PPARγ (hPPARγ) in the CoA-BAP system. A, TBT (▲) showed strong agonistic activity for hRXRα at as low a concentration as that of 9-*cis* retinoic acid (9cRA, ♦). B, TPT (□) showed strong agonistic activity to hPPARγ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

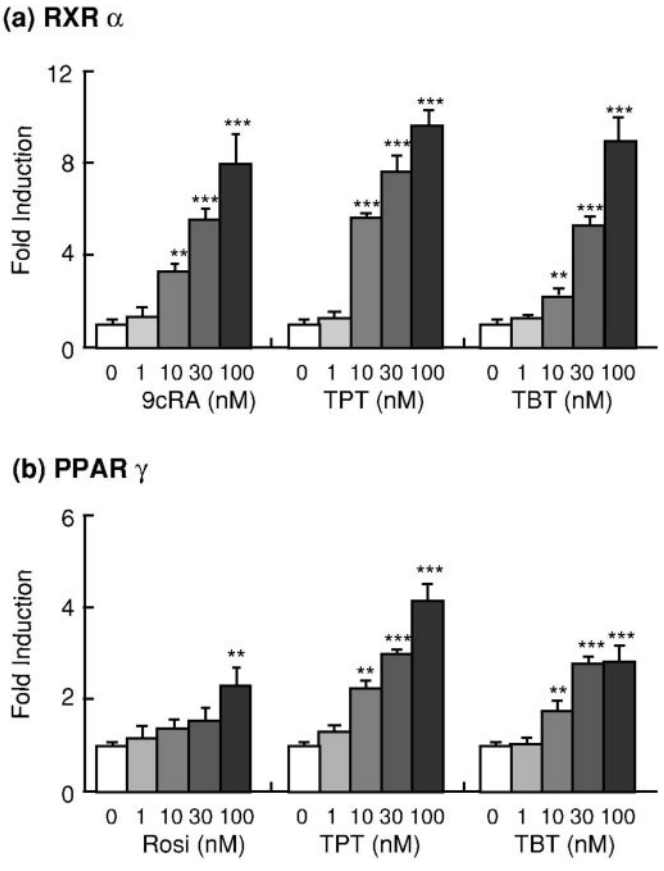

Fig. 4. Organotin compounds induce transcriptional activity through RXRα and PPARγ. Ligand-dependent transactivation of RXRα and PPARγ were detected as luciferase activity. a, F9 cells were cotransfected with a GAL4-DBD-hRXRα expression plasmid and a GAL4-responsive reporter plasmid. b, NIH-3T3 cells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

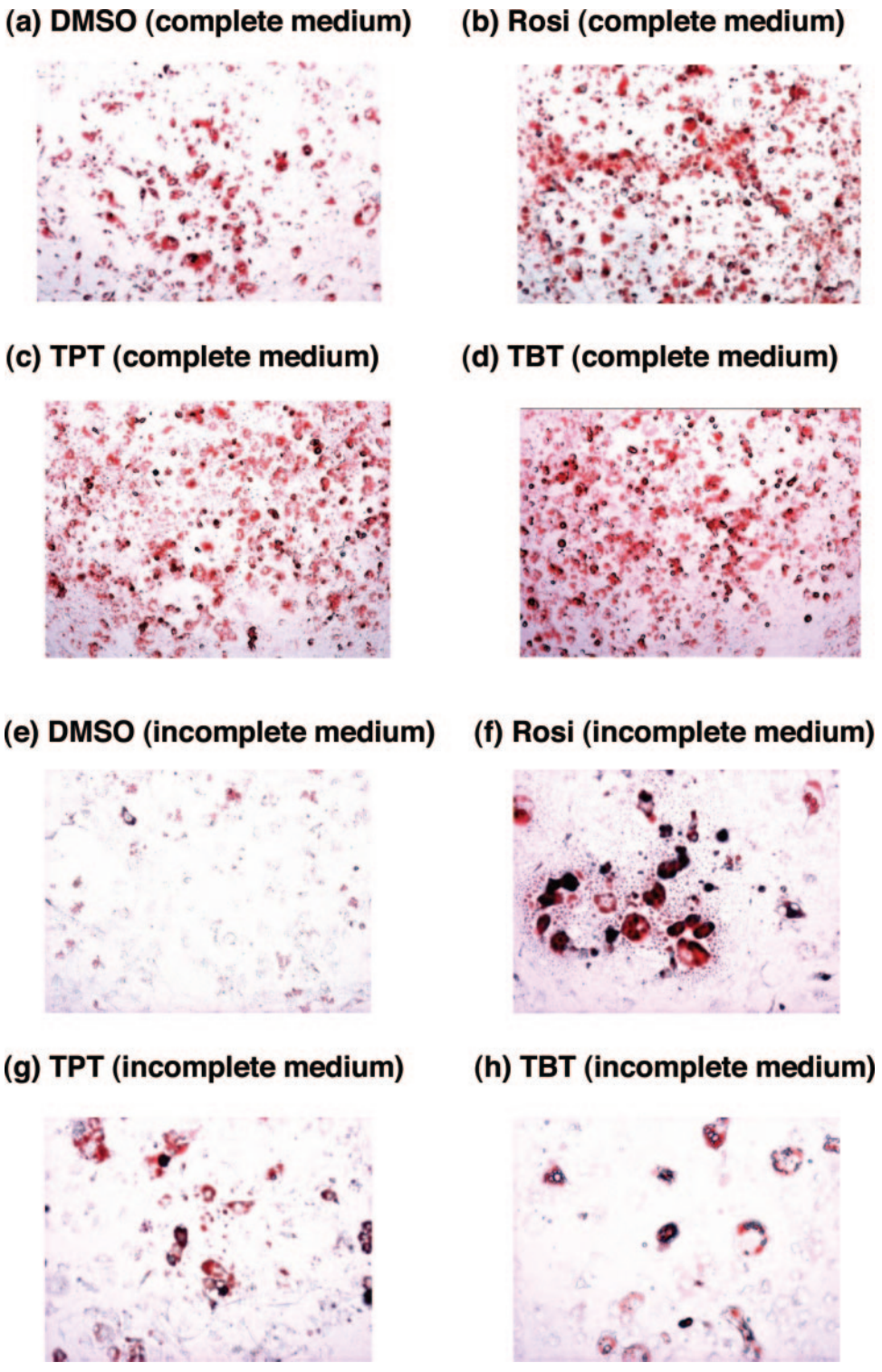

Fig. 5. Enhancement of lipid accumulation by organotin compounds. 3T3-L1 cells were maintained in DMEM containing 10% calf serum. One day after reaching confluence, the cells were treated for 60 h with vehicle only (a and e), 100 nM rosiglitazone (b and f), 100 nM TPT (c and g), or 100 nM TBT (d and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

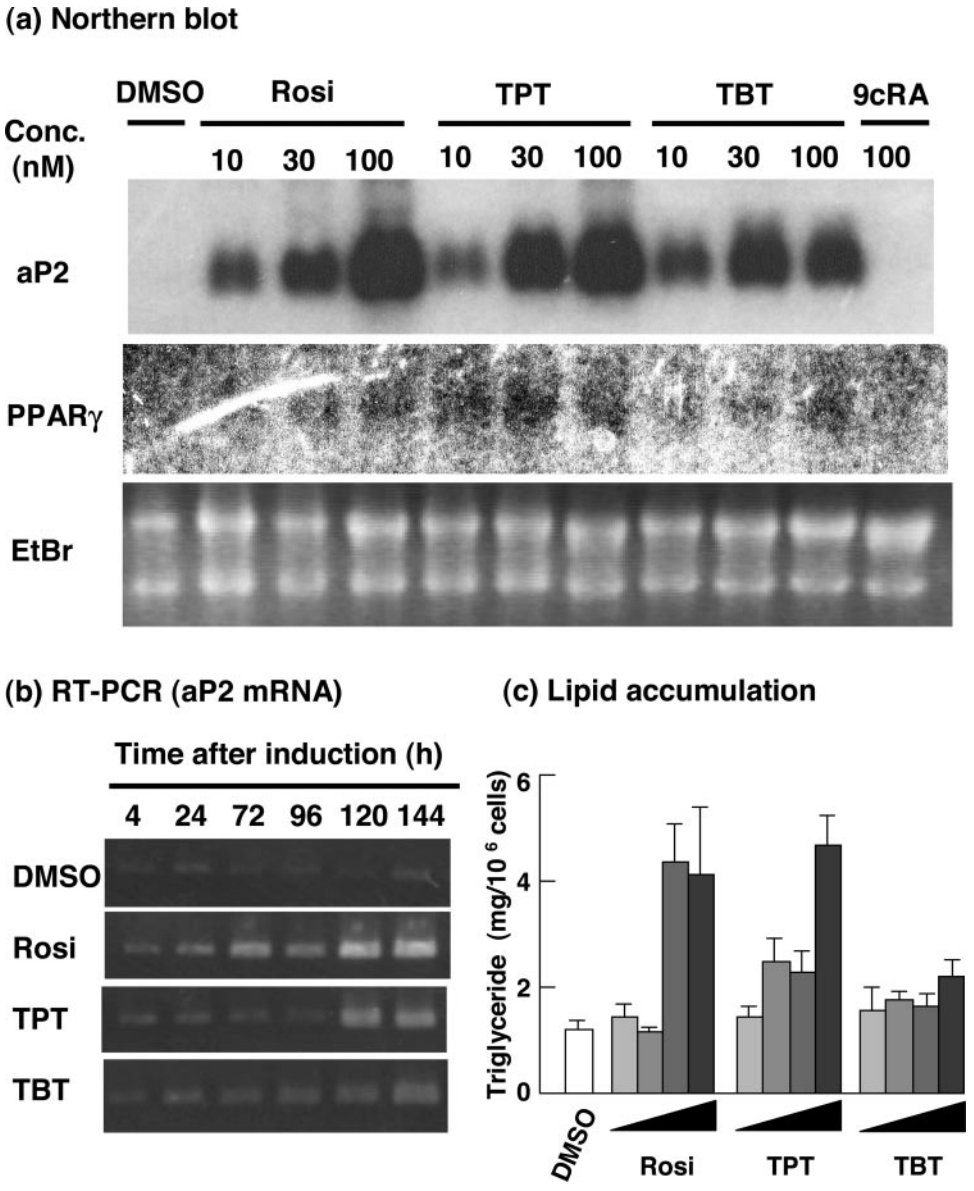

Fig. 6. Induction of adipocyte differentiation markers by organotin compounds. a, induction of adipocyte marker genes by organotin compounds in incomplete differentiation medium. 3T3-L1 cells were maintained in DMEM containing calf serum. One day after reaching confluence, the cells were treated with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

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