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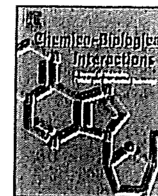

Structure-dependent activation of peroxisome proliferator-activated receptor (PPAR) γ by organotin compounds

Youhei Hiromori [a], Jun-ichi Nishikawa [b], Ichiro Yoshida [a], Hisamitsu Nagase [a], Tsuyoshi Nakanishi [a,c,*]

[a] *Laboratory of Hygienic Chemistry and Molecular Toxicology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu, Gifu, 502-8585, Japan*
[b] *Laboratory of Health Sciences, School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Kyuban-cho, Koshien, Nishinomiya, Hyogo, 663-8179, Japan*
[c] *Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan*

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ABSTRACT

Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) are frequent environmental contaminants and are suspected of disrupting endocrine function in vertebrates and invertebrates. Previously, we reported that TBT and TPT function as powerful agonists for peroxisome proliferator-activated receptor (PPAR) γ and stimulate adipocyte differentiation via the PPARγ signaling pathway. Our current study investigates the structure-dependent binding of butyltin and phenyltin compounds to PPARγ and their ability to activate the receptor. A Scatchard analysis with purified recombinant PPARγ demonstrated that [^{14}C]TPT binds to PPARγ with an equilibrium dissociation constant (K_d) of 66.6 ± 5.2 nM, which approximated the 46.2 ± 2.5 nM K_d of a typical PPARγ agonist, [^{3}H]rosiglitazone (Rosi). TBT, TPT, diphenyltin (DPT), and tetrabutyltin (TeBT) blocked the binding of [^{3}H]Rosi to PPARγ in a competitive manner, and all tested organotin compounds except monobutyltin blocked the binding of [^{14}C]TPT to PPARγ in a competitive manner. Unexpectedly, Rosi did not compete at all with [^{14}C]TPT for binding to PPARγ, and contrary to the results of the competition assay, TBT and TeBT, but not dibutyltin, transcriptionally activated a GAL–PPARγ chimeric receptor. All tested phenyltin compounds transcriptionally activated GAL–PPARγ with an order of potency of TPT > DPT > monophenyltin. In addition, treatment of human choriocarcinoma cells with TBT, TeBT, and all tested phenyltin compounds stimulated production of human chorionic gonadotropin, which is upregulated by PPARγ-mediated transcription. Our observations indicate that trialkylated and triphenylated tin compounds are the most potent PPARγ agonists among the alkylated and phenylated tin compounds, and a phenyl substituent on a tin atom enhances the potency of organotin compounds as a PPARγ agonist much more than a butyl substituent.

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1. Introduction

Organotin compounds have been widely used as antifouling biocides for ships and fishing nets, agricultural fungicides, and rodent repellents [1,2]. These widespread uses have resulted in the release of increasing amounts of organotins into the environment. At very low concentrations, organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) induce irreversible sexual abnormalities in female aquatic invertebrates, particularly marine gastropods, a condition termed "imposex" [3,4]. Organotins may also have various undesirable effects on human health [1,5]. It has been theorized that these compounds act in gastropods as potential competitive inhibitors of aromatase [3,6], which converts androgen to estrogen, resulting in increased levels of unconverted androgens, but their effective concentrations for aromatase inhibition are high [7–9]. In fact, at the same low concentrations needed to induce imposex, these compounds markedly enhance estradiol biosynthesis in human choriocarcinoma cells as well as increasing the activities of both aromatase [9] and 17β-hydroxysteroid dehydrogenase type I (17β-HSD I), which converts the low-activity estrogen estrone to the biologically more active form, estradiol [10]. Despite these reports describing the potential toxicity of organotins in vertebrates and invertebrates, the critical target molecules for the toxicity of organotin compounds remain unclear.

New insight into the mechanism of organotin toxicity is coming from a different direction. Recently, we identified TBT and TPT

Abbreviations: 17β-HSD I, 17β-hydroxysteroid dehydrogenase type I; 9cRA, 9-*cis*-retinoic acid; DBT, dibutyltin; DPT, diphenyltin; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; GST, glutathione S-transferase; hCG, human chorionic gonadotropin; LUC, luciferase; MBT, monobutyltin; MPT, monophenyltin; PPAR, peroxisome proliferator-activated receptor; Rosi, rosiglitazone; PCR, polymerase chain reaction; RXR, retinoid X receptor; TBT, tributyltin; TeBT, tetrabutyltin; TePT, tetraphenyltin; TPT, triphenyltin.

* Corresponding author at: Laboratory of Hygienic Chemistry and Molecular Toxicology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu, 502-8585, Japan. Tel.: +81 58 237 3931; fax: +81 58 237 5979.
E-mail address: nakanishi@gifu-pu.ac.jp (T. Nakanishi).

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as nanomolar agonists for the retinoid X receptor (RXR), which is a member of the nuclear receptor superfamily [11–13]. Functional homologues of RXR have been cloned from Japanese and European gastropods (*Thais clavigera* and *Nucella lapillus*), and TBT binds to both of these homologs at environmentally relevant levels. Furthermore the natural ligand of RXR, 9-*cis*-retinoic acid (9cRA), induces imposex in both species of gastropods [11,14]. Organotin-induced estrogen biosynthesis in human choriocarcinoma cells, including mRNA expression of both aromatase and 17β-HSD I, also depends on the RXR signaling pathway [10,13]. These findings indicate that RXR activation is a critical event in the endocrine disruption caused by TBT and TPT in both gastropods and humans.

TBT and TPT also function as nanomolar agonists for the peroxisome proliferator-activated receptor (PPAR) γ, which is also a member of the nuclear receptor superfamily [12,15]. PPARγ regulates the expression of genes by heterodimerizing with RXR and by binding to the PPAR response elements in the target gene promoter [16,17]. PPARγ is activated by a variety of fatty acids and a class of synthetic antidiabetic agents, the thiazolidinediones [18]. PPARγ agonists such as rosiglitazone (Rosi) are used to treat type II diabetes and reverse insulin resistance in the whole body by sensitizing the muscle and liver tissue to insulin. PPARγ also serves as an essential regulator of adipocyte differentiation and lipid storage in mature adipocytes [19]. Unfortunately, the adipogenic activity of PPARγ may result in undesirable effects such as obesity. Indeed, we [12] and others [15] found that TBT and TPT stimulate adipocyte differentiation *in vitro* and *in vivo*, suggesting that organotin compounds are potential obesogens.

In addition, PPARγ is abundantly expressed in human trophoblast cells and serves as an essential regulator of placental differentiation and endocrine functions, including the production of human chorionic gonadotoropin (hCG) and steroidogenesis in human [20–22]. hCG is a luteotropic factor and stimulation by hCG governs not only progesterone production in the corpus luteum during the first trimester [23] but also testosterone production within the fetal testes [24]. Given the pivotal functional roles of hCG in sexual development and reproduction, factors that change PPARγ-mediated transcription in the placenta may greatly alter fetal development by disrupting these endocrine functions. Indeed, we found that some organotin compounds, including TBT and TPT, stimulate hCG production [9,13]. Activation of PPARγ by TBT and TPT represents a compelling mechanistic example of a class of environmental pollutants that have the ability to affect key adipogenic factors, fat deposit size, and placental functions.

Characterization of organotin compounds as RXR agonists has shown that binding of these organotin compounds to RXR and the responsiveness of the receptor depends on both the number and structure of the alkyl and aryl groups [13]. In addition, these observations suggest that the protein–ligand interactions between organotin compounds and RXR are very different from the interactions between 9cRA and synthetic retinoids [13]. To further our knowledge of how the toxicity of organotin compounds occurs via nuclear receptor signaling, we investigated the structure-dependent binding of butyltin and phenyltin compounds to PPARγ and their ability to activate the receptor. Furthermore, we investigated the correlation between their potency as agonists for PPARγ and hCG production in human choriocarcinoma cells.

2. Materials and methods

2.1. Chemicals

Tin compounds tested in this study were purchased from Tokyo Kasei Kougyo (Tokyo, Japan) or Aldrich Chemicals (Milwaukee, WI) and are listed in Table 1. Rosi was purchased from Cayman Chemical (Ann Arbor, MI). [^{3}H]Rosi (1.85 TBq/mmol) was purchased from American Radiolabeled Chemicals (Saint Louis, MO). [^{14}C]TPT (Tri[U-^{14}C]phenyltin hydroxide; radiochemical purity >96.6%, 2.04 GBq/mmol) was custom-made by Amersham Biosciences (Piscataway, NJ) [13]. All chemicals were dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan). The standardized hCG was a kind gift from Asuka Pharmaceutical (Tokyo, Japan).

2.2. Cell cultures

Cells of the human choriocarcinoma cell line JEG-3 (ATCC No. HTB-36) and Jar (ATCC No. HTB-144) were obtained from ATCC (Manassas, VA). JEG-3 cells were cultured in minimal essential medium (MEM) with 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid solution (Invitrogen, Carlsbad, CA), and 10% fetal calf serum (FCS). Jar cells were cultured in RPMI 1640 medium with 2 mM L-glutamine, 1 mM pyruvate, 4.5 g/L glucose, and 10% FCS. To determine the effect of organotin compounds on reporter gene expression and hCG secretion, the cells were seeded and precultured for 24 h and then treated with either organotin compounds in 0.1% DMSO or vehicle (0.1% DMSO) alone [9,13].

2.3. Plasmid construction

Full-length human PPARγ cDNA was amplified by reverse-transcriptase PCR using total RNA from JEG-3 cells and cloned into pGEX-2T (Amersham Biosciences). The sequences synthesized by PCR were confirmed by DNA sequencing. These constructs were used to generate glutathione S-transferase (GST)–PPARγ fusion proteins. The expression plasmid (pM-mPPAR1) for the ligand binding domain of mouse PPARγ fused to the DNA-binding domain of GAL4 (GAL–PPARγ) and the luciferase (LUC) reporter construct containing four copies of the GAL4 DNA binding site (UAS) followed by the thymidine kinase promoter (p4×UAS-tk-luc) used in the chimeric receptor assay were kind gifts from Dr. Y. Kamei (National Institute of Health and Nutrition, Japan) [12].

2.4. Ligand-binding assay

The GST–PPARγ fusions were expressed in *Escherichia coli* BL21 (DE3) cells and purified by using glutathione-Sepharose 4B (Amersham Biosciences). The purified proteins (30 μg/ml) were incubated with increasing concentrations of either [^{3}H]Rosi or [^{14}C]TPT with or without a 100-fold molar excess of each unlabeled compound. After incubation at 4 °C for 1 h, specific binding was determined with the hydroxyapatite binding assay, as described [13]. Binding in the presence of a 100-fold molar excess of unlabeled ligand was defined as nonspecific binding; specific binding was defined as total binding minus nonspecific binding. Similarly, butyltin and phenyltin compounds were used to compete for [^{3}H]Rosi and [^{14}C]TPT in this assay to determine the binding preferences of PPARγ.

2.5. Transient transfection assay

Transfection was performed with Lipofectamine reagent (Invitrogen) in accordance with the manufacturer's instructions. JEG-3 cells (3×10^4 cells) were seeded in 24-well plates 24 h before transfection with the optimal doses of the pM-mPPAR1 and p4×UAS-tk-luc DNA constructs. At 18 h after transfection, various compounds were added to the transfected cells, which were then cultured in regular culture medium supplemented with 1%

Table 1
Tin compounds tested in this study.

Tin compounds	Abbreviation	Purity (%)	CAS No.[a]	Maximal tested concentration[b]	Source
Butyltin trichloride (monobutyltin)	MBT	>95	1118-46-3	10 μM	Aldrich Chemicals
Dibutyltin dichloride	DBT	>97	683-18-1	100 nM	Tokyo Kasei Kougyo
Tributyltin chloride	TBT	>95	1416-22-0	100 nM	Tokyo Kasei Kougyo
Tetrabutyltin	TeBT	>93	1461-25-2	10 μM	Aldrich Chemicals
Phenyltin trichloride (monophenyltin)	MPT	>98	1124-19-2	10 μM	Aldrich Chemicals
Diphenyltin dichloride	DPT	>96	1135-99-5	1 μM	Aldrich Chemicals
Triphenyltin chloride	TPT	>95	639-58-7	100 nM	Aldrich Chemicals

[a] Chemical Abstracts Service registry number.
[b] Maximal tested concentrations of each tin compounds were defined as a maximal concentration at which the uptake of [3H]thymidine was ≥10% that for the vehicle alone (Ref. [10]).

charcoal-stripped FCS instead of 10% normal FCS. The cells were harvested 30 h later, and extracts were prepared and assayed for *firefly* LUC activity by using the dual-luciferase reporter assay system (Promega, Madison, WI) in accordance with the manufacturer's instructions. To normalize *firefly* LUC activity for transfection and harvesting efficiency, the *Renilla* LUC control reporter construct pRL-TK (Promega) was cotransfected as an internal standard in all reporter experiments. The results are expressed as the average relative *firefly* LUC activity of at least quadruplicate samples.

2.6. Determination of hCG production

hCG production was assessed as previously described [9,13]. In brief, Jar cells were seeded and treated with various compounds in regular culture medium supplemented with 5% charcoal-stripped FCS instead of 10% normal FCS for 48 h. To determine hCG production, the cells were washed and cultured in fresh medium for another 24 h. Culture supernatant was collected, and hCG concentration was determined by ELISA.

2.7. Statistics

All data from the control and treatment groups were obtained from the same numbers of replicated experiments. In addition, all the experiments were carried out independently 2 or 3 times. Dunnett's multiple comparisons test was applied to the raw data using SPSS 12.0J software (Chicago, IL). $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Scatchard analysis of [^{14}C]TPT binding to PPARγ

To directly characterize the binding affinities of butyltin and phenyltin compounds to PPARγ, we performed analyses of the saturation binding of [^{3}H]Rosi and [^{14}C]TPT to chimeric receptors, which consisted of GST fused to PPARγ (GST–PPARγ). Typical binding curves and Scatchard plots for [^{3}H]Rosi and [^{14}C]TPT binding to GST–PPARγ are shown in Fig. 1. The binding of both [^{3}H]Rosi and [^{14}C]TPT to GST–PPARγ was specific and saturating, and no binding was detected in control extracts from bacteria expressing GST (data not shown). Scatchard analyses of the binding of [^{3}H]Rosi to PPARγ yielded a K_d value of 46.2 ± 2.5 nM, which is similar to that previously reported [18], suggesting that this system is useful for determining the binding affinity of organotin compounds to PPARγ. Scatchard analyses of the binding of [^{14}C]TPT to PPARγ yielded a K_d value of 66.6 ± 5.2 nM. Although the K_d value of TPT for PPARγ was approximately 1.5-fold higher than that for Rosi, our results indicate that TPT binds to PPARγ with high affinity in a saturable and specific manner.

3.2. Competition of butyltin and phenyltin compounds with [^{3}H]Rosi and [^{14}C]TPT for binding to PPARγ

To further test which compounds might bind to PPARγ as ligands, we performed competitive ligand-binding assays. We measured the ability of [^{3}H]Rosi or [^{14}C]TPT to compete with the butyltin and phenyltin compounds for binding to GST–PPARγ. Consistent with our previous observations from a reporter gene assay [12], TBT and TPT competed with [^{3}H]Rosi for binding to PPARγ in a concentration-dependent manner (Fig. 2A and B). TPT was a more effective competitor for the binding of [^{3}H]Rosi to PPARγ than Rosi or TBT. In addition, diphenyltin (DPT) competed with [^{3}H]Rosi for binding to PPARγ as well as Rosi did (Fig. 2B). Tetrabutyltin (TeBT) also competed with [^{3}H]Rosi for binding to PPARγ, albeit approximately 10-fold less efficiently than Rosi. Dibutyltin (DBT) competed only slightly with [^{3}H]Rosi (Fig. 2A). Monobutyltin (MBT) and monophenyltin (MPT) failed to compete with [^{3}H]Rosi for binding to PPARγ (Fig. 2A and B).

TBT, TPT, and TeBT also successfully competed with [^{14}C]TPT for binding to PPARγ, and TPT was the most effective competitor among the tested compounds (Fig. 2C and D), consistent with the results of the competitive assay for [^{3}H]Rosi. MBT also failed to compete successfully with [^{14}C]TPT for binding. However, in contrast to the results of the competitive assay for [^{3}H]Rosi, DBT and MPT successfully competed for binding of [^{14}C]TPT (Fig. 2C and D). Unexpectedly, Rosi hardly competed with [^{14}C]TPT for binding. Although DPT competed with [^{14}C]TPT for binding when present at up to 30-fold excess, binding of [^{14}C]TPT increased in the presence of more than 100-fold excess of DPT (Fig. 2D). The high concentration of DPT may have promoted nonspecific binding of [^{14}C]TPT to GST–PPARγ, because same phenomenon was observed using GST instead of GST–PPARγ.

3.3. Activity of butyltin and phenyltin compounds as PPARγ agonists

To identify the functional potency of butyltin and phenyltin compounds as PPARγ agonists, we investigated the ability of butyltin and phenyltin compounds to activate PPARγ. We exposed JEG-3 cells to concentrations of organotins at which the uptake of [^{3}H]thymidine was ≥10% of that previously reported for the vehicle [10], and measured the responsiveness of the PPARγ receptor using a chimeric receptor consisting of the DNA-binding domain of the yeast transcription factor GAL4 and PPARγ (GAL–PPARγ) and a LUC reporter system. GAL–PPARγ binds to the GAL4 UAS in the promoter of the UAS-LUC reporter construct but can activate transcription only in the presence of the hybrid receptor's ligand. A particular advantage of the GAL4 receptor system is that the GAL4 hybrids provide a sensitive and effective means of assaying receptor–ligand interactions, even in the presence of the cells' endogenous wild-type receptors. Indeed, although PPARγ/RXR heterodimers can be fully activated by RXR agonists in addition to PPARγ agonists, we

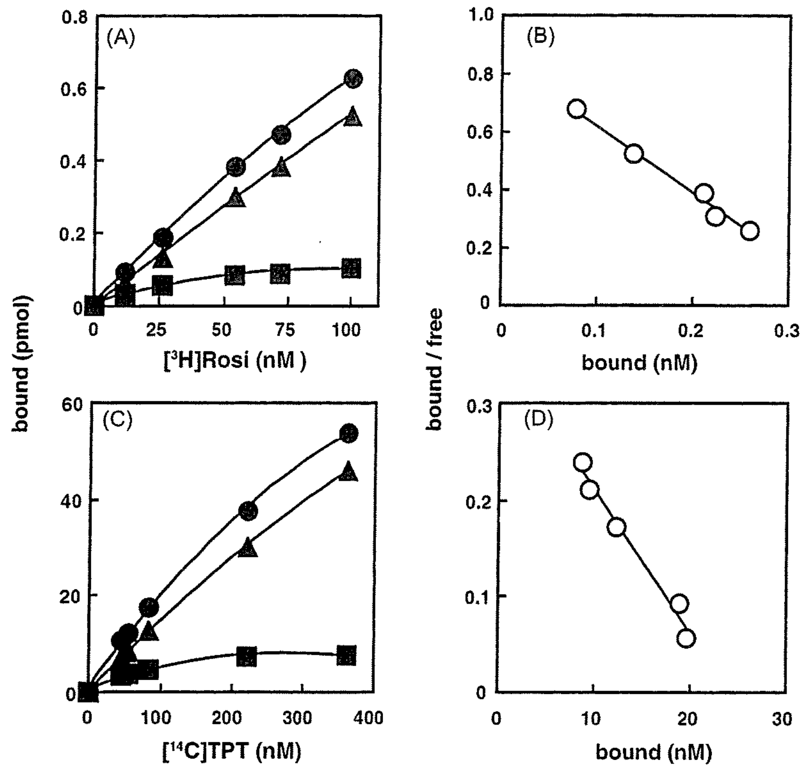

Fig. 1. Saturation binding and Scatchard analysis. (A and C) A GST–PPARγ fusion protein was incubated with increasing concentrations of [^{3}H]Rosi (A) or [^{14}C]TPT (C) in the absence or presence of a 100-fold molar excess of unlabeled Rosi (A) or TPT (C). Specific binding (closed squares) was defined as total binding (closed circles) minus nonspecific binding (closed triangles). (B and D) Scatchard analyses (open circles) were performed on specific binding data (duplicates at each point) for [^{3}H]Rosi (B) and [^{14}C]TPT (D) to yield the K_d values. Similar results were obtained in three independent experiments, and a representative experiment for each receptor–ligand combination is shown.

confirmed that 9cRA, which is a powerful agonist for RXR, failed to induce the expression of LUC in this system. Consistent with our previous observations, 100 nM TBT significantly induced the transactivation function of PPARγ (Fig. 3A, $P < 0.01$), but the level of activation was less than that of Rosi. Although 10 μM TeBT also significantly activated transcription through GAL–PPARγ ($P < 0.05$), the effective concentration was 100-fold higher and the level of activation was only slight compared with that induced by TBT and

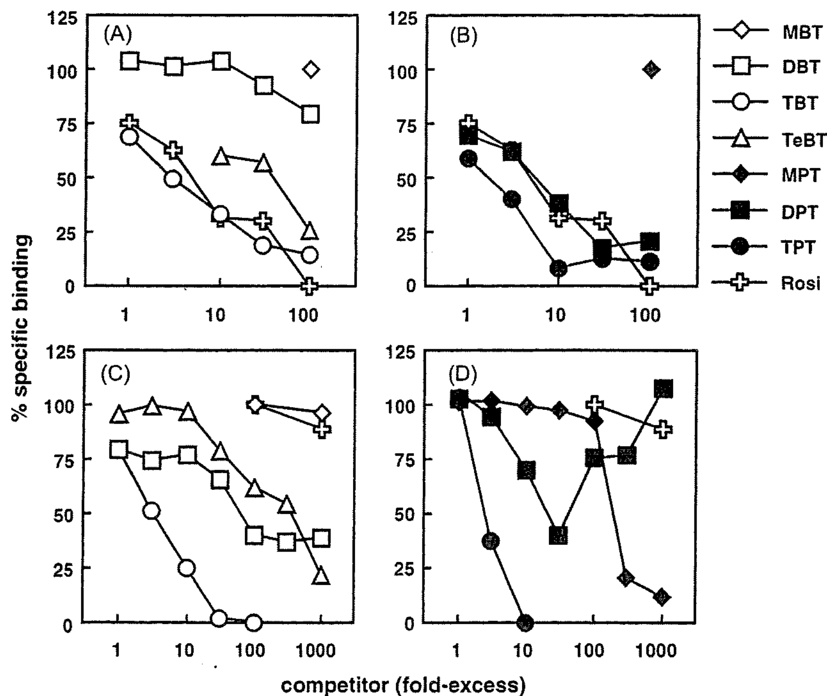

Fig. 2. Competition by Rosi and organotin compounds with [^{3}H]Rosi and [^{14}C]TPT for binding to PPARγ. A GST–PPARγ fusion protein was incubated with increasing concentrations of unlabeled Rosi and butyltins (A and C) or phenyltins (B and D) as competitors in the presence of 50 nM [^{3}H]Rosi (A and B) or 200 nM [^{14}C]TPT (C and D) as ligands. Each experiment was performed at least twice, and representative curves are shown.

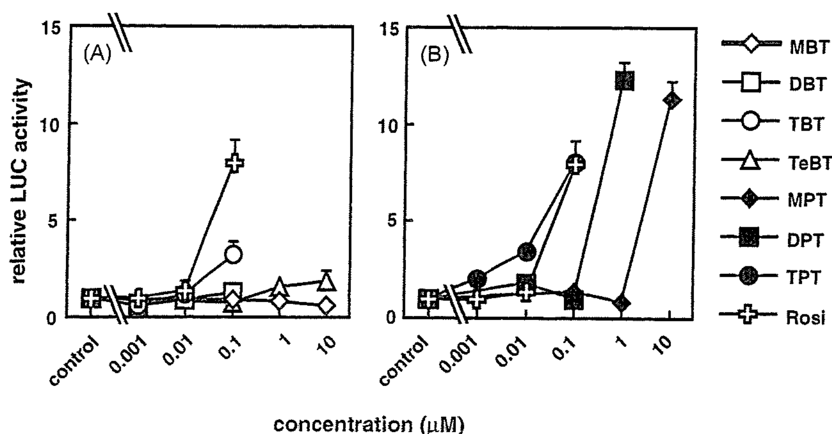

Fig. 3. Ability of organotin compounds to activate GAL–PPARγ. JEG-3 cells were cotransfected with 10 ng p4×UAS-tk-luc and 5 ng pM-mPPAR1 and then treated with Rosi and butyltins (A) or phenyltins (B). pRL-TK (2 ng) was cotransfected as the control for normalization of *Renilla* LUC activity (see Section 2). The results are expressed as average fold activation ± 1 S.D. after normalization to *Renilla* LUC activity.

Rosi (Fig. 3A). MBT and DBT induced no significant activation in the tested range (Fig. 3A).

In contrast, TPT significantly ($P < 0.01$) induced the transactivation function of PPARγ at 1 nM, which is lower than the effective concentration of Rosi (Fig. 3B), suggesting that TPT is a more potential agonist for PPARγ than Rosi. In addition, both 10 μM MPT and 1 μM DPT significantly (both $P < 0.01$) activated PPARγ at levels comparable to that of Rosi, but these concentrations are 100-fold and 10-fold higher, respectively, than the effective concentration of Rosi (Fig. 3B).

3.4. Production of hCG in Jar cells induced by butyltin and phenyltin compounds

We previously investigated the effects of all butyltin and phenyltin compounds tested in the current study on hCG production in human choriocarcinoma cells [9,13]. However, the tested concentrations in the previous study of these compounds, except for TBT and TPT, were too low to evaluate the correlation between their agonistic activity for PPARγ and hCG production. To investigate this correlation, we assessed the effects of these compounds on hCG production in Jar choriocarcinoma cells using the maximum concentrations tested in the experiment shown in Fig. 3. Consistent with our previous observations [9,13], 100 nM TBT and 100 nM TPT dramatically induced hCG production (Fig. 4), and the level of activation was much higher than that induced by Rosi (Fig. 4). hCG production was significantly induced by 10 μM MPT and 1 μM DPT at levels comparable to that of Rosi (Fig. 4). Although much less potent for the transactivation of PPARγ (Fig. 3A), 10 μM TeBT also induced hCG production at a level comparable to that of Rosi (Figs. 3 and 4). However, 10 μM MBT and 100 nM DBT failed to induce hCG production, in agreement with the results of the GAL–PPARγ transactivation assay (Figs. 3 and 4).

4. Discussion

We [11–13,25–27] and others [15] previously showed that TBT and TPT act as novel high-affinity xenobiotic agonists of RXR and PPARγ. This function of organotins was unexpected because the chemical composition and three-dimensional molecular structure of TBT and TPT are different from those of known natural and synthetic nuclear receptor ligands. The ability of tin compounds to bind to and activate RXR activity depends on both the number and length of their alkyl chains [13], but with the exception of a few butyltin compounds [15], the ability of organotins to bind to PPARγ and transactivate the receptor has not been investigated. We show here that the function of butyltin and phenyltin compounds as PPARγ agonists is structure-dependent, similar to their function as RXR agonists.

We first analyzed the K_d value of a typical endocrine-disrupting organotin, TPT, using a custom-made radiolabeled TPT. Our previous observations showed that the K_d values of TPT for RXRα, -β, and -γ were approximately 5–15-fold higher than those of 9cRA, the natural RXR agonist [13]. Here, the K_d value of TPT for PPARγ was comparable to that of Rosi, which is one of the highest-affinity thiazolidinediones [18], suggesting that TPT also binds to PPARγ with high affinity.

Next, we compared butyltins with phenyltins. In competition assays, both TBT and TPT competed with [^{3}H]Rosi and [^{14}C]TPT, but TPT was more competitive for both [^{3}H]Rosi and [^{14}C]TPT than TBT (Fig. 2). Consistent with these results, an approximately 10-fold lower concentration of TPT (10 nM) than of TBT (100 nM) was needed to elicit similar responses (Fig. 3). The di- and monosubstituted organotins DBT and MBT provided no significant activation, whereas DPT and MPT were moderately active in the micromolar range (Fig. 3). In addition, although MPT and MBT did not compete with [^{3}H]Rosi for binding to PPARγ, MPT, but not MBT, competed with [^{14}C]TPT, suggesting that MPT can bind to PPARγ. In our previous observations, both butyltins and phenyltins showed

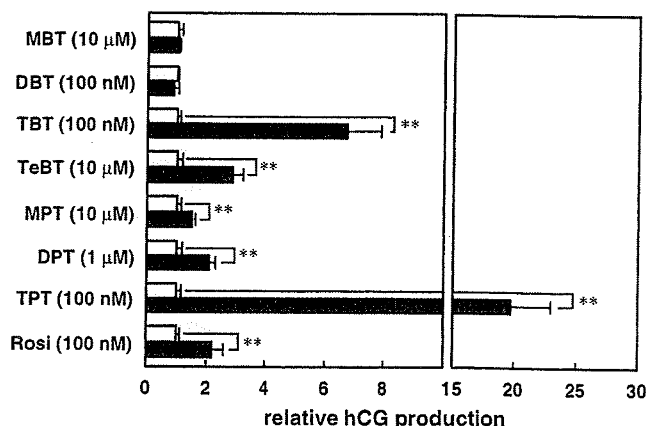

Fig. 4. Ability of organotin compounds to stimulate hCG secretion in Jar cells. Cells were treated with vehicle only (open bars) or the indicated compounds (filled bars) at the maximum concentrations tested in the experiment shown in Fig. 3. Results are expressed as mean ± 1 S.D. of triplicate cultures. The hCG production in vehicle-only cells, calculated from all experiments, was 290.0 ± 85.3 mIU/well/24 h. **$P < 0.01$.

similar potency for RXR [13], but our current results indicate that phenyltins are more potent for both binding and activating PPARγ than butyltins.

On the other hand, DBT competed with [^{14}C]TPT for binding to PPARγ better than MPT (Fig. 2C and D), although DBT failed to activate PPARγ at nanomolar concentrations (Fig. 3A) or at micromolar ranges (data not shown). These observations seem contradictory. However, the cytotoxicity of DBT is much greater than that of MPT because cells did not survive when treated with concentrations of DBT above 1 μM, but survived even when treated with 10 μM MPT [10]. In addition, the binding affinity of DBT for PPARγ is much less than that of TBT, although the cytotoxicity of DBT is greater than that of TBT [10]. Supporting this, we found that TBT was unable to activate PPARγ at concentrations above 500 nM because of its cytotoxicity (data not shown). Consequently, the cytotoxicity of DBT may render DBT-stimulated PPARγ activation undetectable.

The order of potency among the phenyltin compounds was TPT > DPT > MPT according to the results of both the competition and reporter gene assays (Figs. 2 and 3). Tetraphenyltin (TePT) was not tested in the current study because TePT does not dissolve well in DMSO or ethanol, and was therefore difficult to use in the current experiments. Instead, we used TeBT to investigate whether the presence of a fourth alkyl or aryl group would affect the potency as an agonist for PPARγ. Although TeBT significantly transactivated PPARγ, approximately 100-fold higher concentrations were needed than for TBT. In contrast, DBT had no activity for PPARγ (Fig. 3A). In addition, TeBT competed with [^{3}H]Rosi for binding to PPARγ, whereas DBT barely did (Fig. 2A). These results suggest that the presence of a fourth alkyl group on the tin atom decreases the potency of the organotin compounds for PPARγ, and, as with RXR [13], the order of potency is tri- > tetra- > di- > monosubstituted.

The hCG production induced by organotin compounds almost paralleled the results of GAL–PPARγ transactivation (Figs. 3 and 4). This was expected, because PPARγ agonists induce hCG production with a concomitant increase in mRNA expression [20,22]. However, the hCG production induced by 10 μM TeBT was comparable to that induced by 100 nM Rosi (Fig. 4), although TeBT had much lower ability to transactivate PPARγ (Fig. 3A). In addition, 100 nM TBT was more potent than 100 nM Rosi at inducing hCG production, although it was less potent in the transactivation of PPARγ (Figs. 3 and 4). Although these observations seem contradictory, RXR agonists as well as PPARγ agonists induce the production of hCG and mRNA expression of hCGβ (the subunit responsible for the biological specificity of hCG) [28–30] because hCG expression is upregulated by PPARγ/RXR heterodimers, which can be fully activated by ligands for either receptor and are activated synergistically in the presence of ligands for both. Our previous observation indicated that 100 nM TBT and 10 μM TeBT are enough to transactivate RXR [13]. Hence, TBT and TeBT may synergistically activate PPARγ/RXR heterodimers by acting as agonists for both receptors, leading to the induction of hCG production.

Although TPT functions as a high-affinity PPARγ agonist, Rosi did not compete with [^{14}C]TPT at all in our competition assay. Our observations suggest that the protein–ligand interaction between organotins and PPARγ may be markedly different from the interactions between Rosi and PPARγ. For example, in the PPARγ/RXR heterodimer, Rosi binds within the large PPARγ pocket in a U-shaped conformation, with the thiazolidinedione headgroup oriented toward the activation function 2 domain [31]. The thiazolidinedione headgroup makes hydrogen bonds with four residues of PPARγ (S289, H323, H449, and Y473). In contrast, TPT and other agonistic organotin compounds lack a thiazolidinedione headgroup, making their structures very different from that of the thiazolidinediones, such as Rosi. Although further studies are necessary to clarify which amino acids of PPARγ are important for the binding of organotin compounds to the ligand-binding pocket, the ligand–protein contacts of these compounds are most likely unique to each compound.

Human exposure to nonpoint sources of organotins occurs through contaminated dietary sources (seafood and shellfish), as fungicides on food crops, and as antifungal agents in wood treatments, industrial water systems, and textiles [32]. Measured exposure levels of butyltins and phenyltins in human tissue samples are in the range of 3–100 nM [32], high enough for TBT and TPT to activate PPARγ. PPARγ is the target for antidiabetic drugs, but also serves as an essential regulator of adipocyte differentiation and lipid storage in mature adipocytes [19]. Although TBT is suspected to be an environmental obesogen that acts via PPARγ signaling [15], TPT is as prevalent in the environment as TBT but has higher potency and is therefore likely to present an even bigger public health problem.

To our knowledge, this is the first study to characterize butyltin and phenyltin compounds as PPARγ agonists. Our results indicate that the potency of organotin compounds for PPARγ is structure-dependent and that phenyltin compounds are more potent agonists than butyltin compounds. Although many reports have described the potential toxicity of organotins, it remains unclear whether this toxicity involves PPARγ signaling. Therefore, future studies are needed to investigate the precise mechanisms, including PPARγ signaling, underlying the toxicity induced by organotin compounds to distinguish the toxic effects of organotins from similar adverse effects, such as cardiac hypertrophy, edema, hemotoxicity, and hepatotoxicity, that have been associated with existing antidiabetic PPARγ agonists in animal models or in humans [33,34].

Conflict of interest

None declared.

Acknowledgments

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Commentary

Why Public Health Agencies Cannot Depend on Good Laboratory Practices as a Criterion for Selecting Data: The Case of Bisphenol A

John Peterson Myers,[1] Frederick S. vom Saal,[2] Benson T. Akingbemi,[3] Koji Arizono,[4] Scott Belcher,[5] Theo Colborn,[6] Ibrahim Chahoud,[7] D. Andrew Crain,[8] Francesca Farabollini,[9] Louis J. Guillette Jr.,[10] Terry Hassold,[11] Shuk-mei Ho,[12] Patricia A. Hunt,[11] Taisen Iguchi,[13] Susan Jobling,[14] Jun Kanno,[15] Hans Laufer,[16] Michele Marcus,[17] John A. McLachlan,[18] Angel Nadal,[19] Jörg Oehlmann,[20] Nicolás Olea,[21] Paola Palanza,[22] Stefano Parmigiani,[22] Beverly S. Rubin,[23] Gilbert Schoenfelder,[24] Carlos Sonnenschein,[23] Ana M. Soto,[23] Chris E. Talsness,[25] Julia A. Taylor,[2] Laura N. Vandenberg,[23] John G. Vandenbergh,[26] Sarah Vogel,[27] Cheryl S. Watson,[28] Wade V. Welshons,[29] and R. Thomas Zoeller[30]

[1]Environmental Health Sciences, Charlottesville, Virginia, USA; [2]Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA; [3]Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Alabama, USA; [4]Faculty of Environmental and Symbiotic Science, Prefectural University of Kumamoto, Tsukide, Kumamoto, Japan; [5]Department of Pharmacology and Cell Biophysics, Center for Environmental Genetics, University of Cincinnati, Cincinnati, Ohio, USA; [6]The Endocrine Disruption Exchange, Paonia, Colorado, USA; [7]Institut für Klinische Pharmakologie und Toxikologie Charité, Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany; [8]Department of Biology, Maryville College, Maryville, Tennessee, USA; [9]Dipartimento di Fisiologia, Università di Siena, Siena, Italy; [10]Department of Zoology, University of Florida, Gainesville, Florida, USA; [11]School of Molecular Biosciences, Washington State University, Pullman, Washington, USA; [12]Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio, USA; [13]National Institutes of Natural Science, Okazaki Institute for Integrative Bioscience, Bioenvironmental Science, Okazaki, Japan; [14]Department of Biological Sciences, Brunel University, Uxbridge, United Kingdom; [15]Division of Cellular and Molecular Toxicology, National Institute of Health Sciences, Tokyo, Japan; [16]Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut, USA; [17]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; [18]Center for Bioenvironmental Research, Tulane and Xavier Universities, New Orleans, Louisiana, USA; [19]Instituto de Bioingeniería and CIBERDEM, Universidad Miguel Hernández de Elche, Alicante, Spain; [20]Goethe University Frankfurt am Main, Department Aquatic Ecotoxicology, Frankfurt, Germany; [21]Hospital Clínico, CIBERESP, University of Granada, Granada, Spain; [22]Dipartimento di Biologia Evolutiva e Funzionale, Universita' di Parma, Parma, Italy; [23]Tufts Medical School, Boston, Massachusetts, USA; [24]Institute of Pharmacology and Toxicology, University of Wuerzburg, Wuerzburg Germany; [25]Charité University Medical School Berlin, Berlin, Germany; [26]Department of Biology, North Carolina State University, Raleigh, North Carolina, USA; [27]Chemical Heritage Foundation, Philadelphia, Pennsylvania, USA; [28]Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA; [29]Department of Biomedical Sciences, University of Missouri, Columbia, Missouri, USA; [30]Biology Department, University of Massachusetts, Amherst, Massachusetts, USA

BACKGROUND: In their safety evaluations of bisphenol A (BPA), the U.S. Food and Drug Administration (FDA) and a counterpart in Europe, the European Food Safety Authority (EFSA), have given special prominence to two industry-funded studies that adhered to standards defined by Good Laboratory Practices (GLP). These same agencies have given much less weight in risk assessments to a large number of independently replicated non-GLP studies conducted with government funding by the leading experts in various fields of science from around the world.

OBJECTIVES: We reviewed differences between industry-funded GLP studies of BPA conducted by commercial laboratories for regulatory purposes and non-GLP studies conducted in academic and government laboratories to identify hazards and molecular mechanisms mediating adverse effects. We examined the methods and results in the GLP studies that were pivotal in the draft decision of the U.S. FDA declaring BPA safe in relation to findings from studies that were competitive for U.S. National Institutes of Health (NIH) funding, peer-reviewed for publication in leading journals, subject to independent replication, but rejected by the U.S. FDA for regulatory purposes.

DISCUSSION: Although the U.S. FDA and EFSA have deemed two industry-funded GLP studies of BPA to be superior to hundreds of studies funded by the U.S. NIH and NIH counterparts in other countries, the GLP studies on which the agencies based their decisions have serious conceptual and methodologic flaws. In addition, the U.S. FDA and EFSA have mistakenly assumed that GLP yields valid and reliable scientific findings (i.e., "good science"). Their rationale for favoring GLP studies over hundreds of publically funded studies ignores the central factor in determining the reliability and validity of scientific findings, namely, independent replication, and use of the most appropriate and sensitive state-of-the-art assays, neither of which is an expectation of industry-funded GLP research.

CONCLUSIONS: Public health decisions should be based on studies using appropriate protocols with appropriate controls and the most sensitive assays, not GLP. Relevant NIH-funded research using state-of-the-art techniques should play a prominent role in safety evaluations of chemicals.

KEY WORDS: bisphenol A, endocrine disruptors, FDA, Food and Drug Administration, GLP, good laboratory practices, low-dose, nonmonotonic, positive control. *Environ Health Perspect* 117:309–315 (2009). doi:10.1289/ehp.0800173 available via *http://dx.doi.org/* [Online 22 October 2008]

Regulatory agencies in the United States and the European Union (EU) have justified the decision to declare the estrogenic chemical bisphenol A (BPA) safe at current levels of human exposure based on a few studies conducted using Good Laboratory Practices (GLP). In contrast, these agencies have rejected for consideration in their risk assessment of BPA hundreds of laboratory animal and mechanistic cell culture studies conducted by academic and government scientists reporting harm at very low doses of BPA. These studies were rejected primarily because they were not conducted using GLP. We suggest that decisions based on this logic are misguided and will result in continued risk to public health from exposure to BPA, as well as other manmade chemicals.

GLP is a federal rule for conducting research on the health effects or safety testing of drugs or chemicals submitted by private research companies for regulatory purposes. The GLP outlines basic guidelines for conducting scientific research, including the care and feeding of laboratory animals, standards for facility maintenance, calibration and care of equipment, personnel requirements, inspections, study protocols, and collection and storage of raw data (Goldman 1988). These regulations were developed in response to widespread misconduct by private research companies; this misconduct was possible because their data usually do not go through the rigorous, multistage scientific review that is normal for academic data funded by federal agencies and published in the peer-reviewed literature. The lack of these safeguards from academic science had enabled fraud. The U.S. Food and Drug

Address correspondence to J.P. Myers, Environmental Health Sciences, 421 Park St., Charlottesville, VA 22902 USA. Telephone: (434) 220-0348. Fax: (434) 220-0347. E-mail: jpmyers@ehsic.org

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Administration (U.S. FDA) first issued rules for GLP in 1978 after a 2-year federal investigation into sloppy laboratory practices of a number of private research companies (Lublin 1978; Markowitz and Rosner 2002). What began as serious concerns about poor quality research expanded into a criminal investigation of Industrial Bio-Test (IBT), one of the largest private laboratories at the time and a subsidiary of Nalco Chemical Company. In response to the federal investigation, the U.S. Environmental Protection Agency (EPA) demanded that 235 chemical companies reexamine the > 4,000 tests conducted by the laboratory. In 1983, three men from IBT were found guilty of deliberating doctoring data and were sentenced to prison (Lublin 1978; Markowitz and Rosner 2002). The fraudulent practices of IBT brought into question 15% of the pesticides approved for use in the United States. That same year, the U.S. EPA issued similar GLP rules for regulatory testing.

Both the U.S. FDA (2008a) and European Food Safety Authority (ESFA 2006) have recently published documents demonstrating that their decision to continue to declare BPA safe at current exposure levels was based primarily on the results of a few industry-funded studies that followed GLP guidelines. These decisions stand in stark contrast to the decisions concerning the potential risks to human health reached by a panel of 38 experts at a U.S. National Institutes of Health (NIH)-sponsored conference, who published The Chapel Hill Consensus Statement (vom Saal et al. 2007), as well as five review articles (Crain et al. 2007; Keri et al. 2007; Richter et al. 2007a; Vandenberg et al. 2007a; Wetherill et al. 2007). These peer-reviewed articles covered approximately 700 articles concerning BPA and represented a comprehensive review of the literature as of the end of 2006. In addition, the U.S. FDA draft decision contradicted the conclusions reached by the National Toxicology Program (NTP), which had spent 2 years investigating this question (NTP 2008). An important role of the NTP is to advise the U.S. FDA about the science relating to toxic chemicals in food, but in an unusual move, the U.S. FDA chose to release its draft report before the release of the final report on BPA by the NTP and without indicating who at the U.S. FDA was involved in preparing the draft report (U.S. FDA 2008b). At a hearing on 16 September 2008 regarding the draft report on BPA, the U.S. FDA announced that their goal was to have a subcommittee of the U.S. FDA Science Board complete a review of the draft decision by the end of October 2008. This would presumably also involve review by the subcommittee members of the approximately 1,000 articles relating to BPA.

We believe that the methods employed in chemical industry–sponsored GLP studies are incapable of detecting low-dose endocrine-disrupting effects of BPA and other hormonally active chemicals. Detecting endocrine-disrupting effects at low doses of chemicals such as BPA requires sophisticated and modern assays and analyses that have been developed in advanced, usually federally funded laboratories over the past decade. This is especially apparent when one examines what is now known about functional effects of BPA on a wide range of end points (Richter et al. 2007a; Welshons et al. 2006; Wetherill et al. 2007). These end points include those mediated by recently discovered estrogen response pathways initiated in human and animal cell membranes (nonclassical or alternative estrogen response mechanisms), which multiple laboratories have shown to be equally sensitive to BPA and estradiol in terms of activating effects in human and animal cells at low picomolar through low nanomolar concentrations (Alonso-Magdalena et al. 2008; Wetherill et al. 2007; Wozniak et al. 2005; Zsarnovszky et al. 2005).

The effects of BPA documented in these studies include a diverse array for which there are no data from GLP studies because the end points have not been examined: altered metabolism related to metabolic syndrome (Alonso-Magdalena et al. 2005, 2006, 2008; Ropero et al. 2008); altered adiponectin secretion (Hugo et al. 2008), which is a condition predicting heart disease and type 2 diabetes (Lang et al. 2008); altered epigenetic programming leading to precancerous lesions of the prostate (Ho et al. 2006); differential growth patterns in the developing prostate (Timms et al. 2005); abnormal growth, gene expression, and precancerous lesions of the mammary glands (Soto et al. 2008); and adverse effects on the female reproductive system, including uterine fibroids, paraovarian cysts, and chromosomal abnormalities in oocytes (Newbold et al. 2007; Susiarjo et al. 2007). There is also a large literature on neuroanatomic, neurochemical, and behavioral abnormalities caused by low doses of BPA (Leranth et al. 2008; Richter et al. 2007a), which also are not capable of being detected by current GLP studies conducted for regulatory purposes because of their out-of-date assays.

The approaches used by academic and government scientists to study the potential health hazards of BPA contrast sharply with those still used by the chemical industry that are relied on by regulatory agencies in the United States and Europe, including the two studies identified by both the U.S. FDA and European Food Safety Authority (EFSA) as central to the decision to declare BPA safe at current human exposure levels (Tyl et al. 2002, 2008a). By using outdated and insensitive assays that were supposed to have been replaced by a new battery of screens and tests by 2000 [as mandated by the U.S. Congress in 1996 in the Food Quality Protection Act (1996), but which has, as yet, still not occurred], these studies conducted using GLP fail to find any adverse effects.

Reliability and Validity

Reliability and validity are separate issues, although in the experimental research described here, validity and reliability basically refer to research that is credible. Golafshani (2003) noted that "reliability" refers to the extent to which results are consistent over time and are an accurate representation of the total population under study. Of central importance is that the results of a study must be reproduced under a similar methodology to be considered to be reliable. "Validity" refers to whether the research measures what it was intended to measure, and valid findings are considered to be true. In other words, reliability is determined by whether the results are replicable, whereas validity is assessed by whether the methods used result in finding the truth as a result of the investigator actually measuring what the study intended to measure.

Use of GLP in Regulatory Decision Making

Despite strong evidence of aberrations caused by low doses of BPA in animals exposed during fetal and neonatal life in studies conducted by the world's leading academic and government experts in the fields of endocrine disruption, endocrinology, neurobiology, reproductive biology, genetics, and metabolism, a relatively small number of studies reporting no adverse effects at low doses of BPA have continued to be promoted by the chemical industry and used by regulatory agencies (e.g., Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2002, 2008a). According to the U.S. FDA, these are accepted because they used GLP (U.S. EPA 2008), with the implication that studies not employing GLP are not reliable or valid (U.S. FDA 2008a).

GLP does not guarantee reliability or validity of scientific results. Unfortunately, although GLP creates the semblance of reliable and valid science, it actually offers no such guarantee. GLP specifies nothing about the quality of the research design, the skills of the technicians, the sensitivity of the assays, or whether the methods employed are current or out-of-date. (All of the above are central issues in the review of a grant proposal by an NIH panel.) GLP simply indicates that the laboratory technicians/scientists performing experiments follow highly detailed U.S. EPA requirements [or in the EU, Organization for Economic Co-operation and Development (OECD) requirements] for record keeping, including details of the conduct of the

experiment and archiving relevant biological and chemical materials (U.S. EPA 2008).

These record-keeping procedures in GLP were instituted because of widespread misconduct being committed by commercial testing laboratories (described above). These fraudulent results were possible because contract laboratory studies used in the regulatory process are rarely subject to the checks and balances that peer-reviewed, replicated scientific findings undergo. Without that acid test of reliability (replication by other independent scientists), other procedures were needed. Hence GLP was implemented, despite its severe limitations.

NIH-funded research subject to more stringent reviews than GLP. Although few NIH-funded investigators adhere to GLP-mandated record keeping, the procedures of GLP are actually surpassed by the procedures required for NIH-funded science published in peer-reviewed journals. NIH-funded studies pass through three phases of peer review that are far more challenging than GLP requirements. First, the principal scientists must have demonstrated competence to conduct the research, and experimental methods, assays, and laboratory environment must involve use of state-of-the-art techniques to be competitive for NIH funding. Second, results are published in peer-reviewed journals, with detailed evaluations by independent experts examining all aspects of the study. And third, the findings are challenged by independent efforts to replicate; for example, the initial findings concerning the stimulating effects of estrogenic chemicals on the mouse prostate (Nagel et al. 1997; vom Saal et al. 1997) were independently replicated and extended by Gupta (2000), which led to an editorial identifying "initial results confirmed" (Sheehan 2000).

Typically, within a laboratory, interesting findings are also followed by subsequent publications extending the prior findings; examples include the findings of BPA effects on β cells in the mouse pancreas (Alonso-Magdalena et al. 2005, 2006, 2008) and the effects of estrogenic chemicals and drugs on the developing mouse prostate that followed earlier findings (described above) from this same group (Timms et al. 2005; Richter et al. 2007b). In particular, independent replication by competent, respected scientists is the main criterion of acceptance of the findings as having been demonstrated to be reliable and having been validated by virtue of coming to the same conclusion using a variety of sophisticated techniques in multiple publications.

An important criticism of the approach taken by the U.S. FDA in its assessment of the now approximately 1,000 articles on BPA is that it appears to have made no attempt to connect the dots between replicated studies; instead, the U.S. FDA appears to have assessed each study without regard to whether it had been confirmed by other studies.

Thus, collectively, many phases used to verify the reliability and validity of NIH-funded published research have been completely ignored by the U.S. FDA, whereas industry-funded GLP research is rarely, if ever, subject to these central requirements and yet is accepted by regulatory agencies as reliable and valid.

The U.S. FDA's misguided gold standard. In this light, the U.S. FDA's reliance upon GLP as the gold standard is scientifically misguided. Furthermore, U.S. FDA administrators are ignoring published critiques of the GLP studies it considers reliable and valid, such as the study by Tyl et al. (2002) and two coordinated studies conducted at the same time by Ashby et al. (1999) and Cagen et al. (1999). Each was an industry-funded study conducted using GLP. Each was harshly criticized in peer-reviewed publications by academic scientists and government panels [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2007; NTP 2001; vom Saal and Hughes 2005; vom Saal and Welshons 2006]. Yet, the U.S. FDA and EFSA panels still assert that these studies represent the gold standard in toxicologic research.

Specifically, the studies of Cagen et al. (1999) and Ashby et al. (1999) were recently rejected by the NTP CERHR panel on BPA as unusable for consideration in its evaluation of the health hazards posed by BPA (CERHR 2007). Both the Ashby et al. (1999) and Cagen et al. (1999) studies reported finding no effect of their positive control [the estrogenic drug diethylstilbestrol (DES)] on any outcome, although these failures were not acknowledged by the authors in either article. In experimental science, the failure of a positive control to show an effect indicates the experiment failed, which is the conclusion reached by the CERHR panel (CERHR 2007).

The Tyl et al. 2002 study, which the U.S. FDA still accepts as a major study for determination of the safety of BPA (U.S. FDA 2008a, 2008b), was criticized by an NTP panel that met in 2000 to examine the low-dose issue (NTP 2001), as well as in subsequent publications (vom Saal and Hughes 2005; vom Saal and Welshons 2006), for using an insensitive rat (the CD-SD rat) that requires extremely high doses (≥ 50 μg/kg/day) of the potent estrogenic drug ethinylestradiol to show effects such as those examined in the study by Tyl et al. (2002). This dose of ethinylestradiol is > 100 times higher than the approximately 0.3 μg/kg/day used by women in oral contraceptives. The fact that Tyl et al. (2002) adhered to GLP did not protect them from using insensitive animals. This led the NTP (2001) to state:

> Because of clear species and strain differences in sensitivity, animal model selection should be based on responsiveness to endocrine-active agents of concern (i.e., responsive to positive controls), not on convenience and familiarity.

Thus, when reviewed by other scientists, three prior major GLP studies of BPA have been found to be so flawed as to be useless for guiding regulatory agencies in decision making. A new GLP study has now been published by Tyl et al. (2008a). Close examination of this study also reveals fatal flaws which render it useless for regulatory purposes, even though it conforms to GLP.

Examples of Flaws Ignored by the U.S. FDA and EFSA in a Recent GLP Study of BPA

In summary, the flaws in Tyl et al. (2008a) are as follows:

- The high dose required for the positive control (estradiol) to cause an effect means the system used by Tyl et al. (2008a), at least in her laboratory, is relatively insensitive to exogenous estrogens and thus inappropriate for studying low-dose effects of estrogenic compounds such as BPA. The lack of response to low doses of estradiol or BPA in the Tyl laboratory is puzzling, in that the strain of mice used in these experiments (the CD-1 mouse) has been reported in > 20 other peer-reviewed publications to show adverse effects in response to very low doses of BPA (vom Saal 2008), as well as many other studies showing low-dose effects in response to the natural hormone estradiol, the estrogenic drugs ethinylestradiol and DES, and to other estrogenic chemicals.
- Tyl et al. (2008a) used insensitive, out-of-date protocols and assays that are incapable of finding many of the adverse effects reported by more sophisticated studies conducted by independent NIH-funded scientists as well as scientists funded by government agencies in other countries.
- In the specific case of testing for changes in prostate weight, Tyl et al. (2008a) reported an abnormally high prostate weight for control animals that exceeds by > 70% the prostate weights reported by other studies for animals of the same strain and similar age (e.g., Gupta 2000; Ruhlen et al. 2008). This suggests that the dissection procedures for the prostate in the Tyl laboratory included other nonprostatic tissues in the weight measurements, rendering them unusable for studying weight changes in the prostate in response to BPA or estradiol; neither chemical showed any effect on the selected end points, which directly contradicts other findings concerning opposite effects of low and high doses of estrogen on the prostate (Putz et al. 2001; Timms et al. 2005; vom Saal et al. 1997).

Aberrant insensitivity of CD-1 mouse to estrogens. Tyl et al. (2008a) used estradiol as a positive control. It was fed to female mice before and during pregnancy and lactation at 80–220 μg/kg/day; after weaning, estradiol was fed to offspring at doses of 80–100 μg/kg/day. Estradiol was used as a positive control because BPA is a man-made endocrine-disrupting estrogenic chemical.

Many published findings reporting effects of very low doses of positive control estrogens and BPA in CD-1 mice demonstrate that the CD-1 mouse was somehow rendered insensitive in the test system used by Tyl et al. (2008a). The fact that a dose of 100–200 μg/kg/day estradiol was necessary to show an effect of the positive control predicts that Tyl et al. (2008a) should not detect effects of BPA < 10–100 mg/kg/day, far above the low-dose range relevant to human exposures that was supposedly of interest.

For nuclear estrogen receptor–mediated effects via regulation of gene activity (nuclear estrogen receptors are transcription factors whose activity is regulated by binding to estrogen), prior studies have typically shown a 1,000-fold lower activity for BPA relative to estradiol or potent estrogenic drugs, including DES and ethinylestradiol. For example, Richter et al. (2007b) reported an increase in androgen receptor gene activity to estradiol at 1 pM (0.28 pg/mL) in fetal CD-1 mouse prostatic mesenchyme cells in primary culture, and the same response was found for BPA at 1,000 pM (228 pg/mL); the *in vitro* response to estradiol was predicted by the response of the prostate to increasing free serum estradiol from 0.2 to 0.3 pg/mL in male mouse fetuses via estradiol administration to the mother (vom Saal et al. 1997). Other research showed that a significant effect on development of the male reproductive system in CF-1 mice occurred at a maternal dose of 0.002 μg/kg/day ethinylestradiol (Thayer et al. 2001), similar to effects observed with 2–20 μg/kg/day BPA (vom Saal et al. 1998). The research of Honma et al. (2002) showed accelerated puberty in CD-1 (ICR) mice at a DES dose of 0.02 μg/kg/day (the positive control), and the same response to BPA occurred at 20 μg/kg/day, again revealing a 1,000-fold difference between the positive control estrogen and BPA.

There are many other examples of findings where a higher dose of BPA was required to cause the same effect as the positive control estrogen (estradiol, ethinylestradiol, or DES) in studies where the effects were mediated by the classical nuclear estrogen receptors, in contrast to the more recently discovered rapid signaling estrogen response system where BPA and these positive control estrogens have equal potency, as described above. In summary, CD-1 mice have been used by a large number of academic and government investigators and have been reported in peer-reviewed publications to be sensitive to positive control estrogens within the range of human sensitivity based on *in vivo* and *in vitro* studies via the classical estrogen receptor α–mediated response mechanism. The CD-1 mouse is the animal model that has been used by the U.S. National Institute of Environmental Health Sciences (NIEHS) for decades, because it is considered the best animal model for predicting the effects of developmental exposure to estrogen in humans (Newbold 1995; Newbold et al. 2007).

The failure of traditional toxicologic studies conducted by Tyl et al. (2008a, 2008b) to detect the wide range of adverse effects of even relatively high doses of BPA or of low doses of estradiol that have been reported in numerous studies by academic and government scientists provides evidence that the GLP protocols established long ago by regulatory agencies to determine the toxicity of chemicals are inappropriate for detecting the endocrine-disrupting activities of chemicals such as BPA. Indeed, this was the premise of the congressional mandate in the Food Quality Protection Act (1996) for the U.S. EPA to establish a new set of assays for endocrine-disrupting chemicals, although this process has been systematically delayed and is > 8 years behind the congressionally mandated date of 2000 to have these new assays validated.

Citing Tyl et al. (2008a), the EFSA report on BPA (EFSA 2006) stated that "the positive control substance, 17β-estradiol, resulted in reproductive and developmental toxicity." This report failed to acknowledge that only a very high dose of the positive control was sufficient to elicit effects and that this meant that the experiments conducted in the Tyl laboratory were for some reason very insensitive to any estrogen and thus inappropriate for use in a study to examine low-dose estrogenic effects of BPA.

Based on the preliminary report released by the U.S. FDA regarding BPA (U.S. FDA 2008a), it appears that the U.S. FDA has followed the lead of the EFSA in its lack of understanding of the importance of the dose of the positive control estrogen required to cause adverse effects. The consequence is that the U.S. FDA has relied primarily on the study of Tyl et al. (2008a, 2008b), with the result that the U.S. FDA has assured Americans that BPA is safe at current human exposure levels.

Several factors might account for the insensitivity of the CD-1 mouse in the Tyl et al. studies (2008a, 2008b) conducted at Research Triangle Institute (RTI), a testing facility that conducted these (as well as previous) studies funded by the American Chemistry Council. One possibility is that the diet used in these studies may have interfered with the results. The feed used by Tyl et al. (2008a) in this experiment (Purina 5002) has been shown by others to interfere with responses to exogenous estrogenic chemicals, blocking adverse effects documented on other diets. For example, a number of years ago, Thigpen et al. (2003) at the NIEHS recommended against the use of Purina 5002 in studies of endocrine-disrupting chemicals. Tyl et al. (2008a) measured some specific phytoestrogens in Purina 5002 feed by chemical analysis; however, in a report on NIH-sponsored meetings on this subject, Heindel and vom Saal (2008) pointed out that this is an insufficient control for total dietary estrogenic contaminants that can disrupt studies involving the effects of estrogenic chemicals.

A second possibility is that there are strain differences in sensitivity developed in the CD-1 mouse sold by the various Charles River Laboratories located in different regions. We consider this unlikely, because most laboratories regularly replace their CD-1 mouse breeder stock from Charles River Laboratories, and practices there make it unlikely that the sensitivity of this outbred stock to estrogens has changed dramatically over a very short period of time. Also, because RTI, where the Tyl studies were conducted, is very near the laboratories of the NIEHS, it is likely that the CD-1 mice used by these two programs were purchased from the same breeding facility.

Use of insensitive, out-of-date protocols and assays. Another serious concern about the two recent studies by Tyl et al. (2008a, 2008b) is the experimental approach used, thus raising questions about the validity of the studies. The study design used by Tyl et al. (2008a, 2008b) has been superseded by advances in both experimental design and analytical tools developed by NIH-funded scientists (and their counterparts in Europe and Asia) since the mid-1990s. The methods used by Tyl et al., primarily wet weight changes of tissues, gross histologic changes, and developmental landmarks such as vaginal opening, were established procedures by the 1950s. Thus, a major limitation of the Tyl studies is the failure to measure more meaningful and sensitive end points in order to detect the effects of low-dose BPA exposure, which are often not macroscopic in nature. Indeed, in 2001, the director of the reproductive division of the National Health and Environmental Effects Research Laboratory at the U.S. EPA stated that the inconclusive results concerning effects of BPA on reproductive toxicology can only be solved by understanding the mechanisms (Triendl 2001). With current GLP standards it is not possible to study mechanisms because they still rely on out-of-date assays.

As one example of a comparison between the approach by Tyl et al. (2008a) and independent government-funded academic scientists, extensive research has been conducted by Soto et al. (2008) and by other independent academic and government scientists

describing effects of exposure of female mice and rats to very low doses of BPA during perinatal development on the mammary glands (Jenkins et al. 2009). Although Tyl et al. (2008a) reported no low-dose effects of BPA on the mammary glands using conventional histologic analysis, there have been consistent findings of adverse effects of low doses of BPA from studies that used more sophisticated and sensitive analysis of whole mounted mammary glands to facilitate detection of microscopic lesions, coupled with immunostaining for regulatory proteins as well as techniques for determination of aberrant gene expression associated with progression to cancer. These peer-reviewed studies have reported detecting changes during embryonic development of mammary glands as well as abnormalities detected during adolescence through adulthood that are indicative of mammary gland cancer as well as other developmental abnormalities (Colerangle and Roy 1997; Durando et al. 2007; Jenkins et al. 2009; LaPensee et al. 2008; Markey et al. 2001, 2005; Moral et al. 2008; Munoz-de-Toro et al. 2005; Murray et al. 2007; Nikaido et al. 2004; Vandenberg et al. 2006, 2007b; Wadia et al. 2007).

Similar to the findings for the mammary gland, Ogura et al. (2007) reported that if tissues were analyzed by conventional histologic methods (staining with hematoxalin and eosin), prenatal exposure to low doses of BPA or DES showed no effects on prostate development, whereas if the sections were analyzed using antibodies that identified basal cells and basal cell squamous metaplasia, then significant effects were revealed. Squamous metaplasia of basal cells indicates abnormal proliferation and function of the prostate stem cell population that is thought to transform into neoplastic cells; Ho et al. (2006) reported that neonatal exposure to very low doses of BPA caused 100% of male rats to develop high-grade prostatic intraepithelial neoplastic lesions later in life. All of these studies were rejected by the U.S. FDA as not adequate for making regulatory decisions about the safety of BPA. Instead, the U.S. FDA relied upon Tyl et al. (2008a), even though the study used techniques that Ogura et al. (2007) showed lacked the sensitivity of 21st century experimental approaches.

Although findings regarding changes in brain structure, brain chemistry, and behavior represent the largest portion of the literature on low-dose BPA, Tyl et al. (2008a) did not examine any neurobehavioral end points. The NTP (2008) and the NIEHS conference consensus reports (vom Saal et al. 2007) both indicated concern about neurobehavioral effects of low doses of BPA. Thus, the absence of studies that included neurobehavioral end points is a glaring omission of Tyl et al. (2008a, 2008b).

Flawed prostate dissection. Data presented by Tyl et al. (2008a) raise questions about the adequacy of techniques used in their BPA studies. Specifically, Tyl et al. (2008a) reported that the prostate in 3.5-month-old control male CD-1 mice weighed > 70 mg [see Table 3 in Tyl et al. (2008a) for data on F_1 retained males]. This average control weight contrasts sharply with those reported from other laboratories. Specifically, the weight of the prostate in 2- to 3-month-old CD-1 mice using the dissection technique based on both Ruhlen et al. (2008) and Gupta (2000) and at the NIEHS (Newbold RR, personal communication) is about 40 mg. Several studies have reported that prenatal exposure to very low doses of BPA and positive control estrogens increased prostate size, prostatic androgen receptors, and prostate androgen receptor gene activity (Gupta 2000; Richter et al. 2007b; Thayer et al. 2001; Timms et al. 2005; vom Saal et al. 1997), but the enlarged prostate of experimental animals exposed to BPA in these laboratories weighed less than the prostates in the control animals of Tyl et al. (2008a). This raises serious questions about the procedures and/or animals used by Tyl et al. The weight of prostate reported by Tyl et al. (2008a) suggests that the technique used for dissecting the prostate resulted in nonprostatic tissue being weighed along with prostate. The seminal vesicle, coagulating gland, and dorsolateral prostate all merge together where the ejaculatory ducts enter the urethra, and there are also fat deposits on the prostate. This poses a challenge for those without proper training in distinguishing these different tissues during dissection in mice.

Alternatively, as male rodents age, they are prone to develop prostatitis. Although this inflammatory disease leads to an increase in prostate size and could thus account for the very large prostate weights reported by Tyl et al. (2008a), anyone familiar with the appearance of prostatitis would detect this abnormality upon histologic examination, which Tyl et al (2008a) supposedly conducted. Also, prostatitis is rare in young-adult mice or rats (Cowin et al. 2008), and the size of the prostates in the Tyl et al. (2008a) study were similar to those for middle-aged and old male mice.

The findings regarding effects of BPA on the prostate presented by Tyl et al. (2008a) are thus suspect and cannot be used as evidence that other earlier studies (Gupta 2000; Timms et al. 2005; vom Saal et al. 1997) are not replicable. Given these problems in prostate weight measurements, it is not surprising that even very high doses of BPA or estradiol reported by Tyl et al. (2008a) had no effect on the prostate, in sharp contrast to other studies that showed stimulation of the prostate at low doses of estrogen and inhibition at high doses (Putz et al. 2001; Timms et al. 2005).

In addition to the problem associated with the high prostate weight reported by Tyl et al. (2008a), in a separate measurement the authors combined the anterior prostate (coagulating gland) and seminal vesicle, presenting these two organs as one combined outcome measure. This is wrong and misleading. The coagulating glands emerge as the anterior ducts of the prostate from the dorsocranial region of the urogenital sinus, whereas the seminal vesicles bud from the proximal region of the Wolffian ducts. Elevated estrogen is associated with an increase in prostate size associated with an increase in prostate androgen receptors, whereas a decrease in seminal vesicle size is associated with a reduction in 5α-reductase, an enzyme that converts testosterone to the more potent androgen 5α-dihydrotestosterone (Nonneman et al. 1992). Low doses of BPA have been shown to decrease the size of organs that differentiate from the embryonic Wolffian ducts (epididymides and seminal vesicles) while increasing the size of regions of the prostate that develop from the urogenital sinus (vom Saal et al. 1998). Combining these different organs (it is technically not difficult to separate them) was thus inappropriate because they develop from different embryonic tissues that show markedly different responses to estrogenic chemicals during development. In fact, Ogura et al. (2007) reported that the anterior prostate (coagulating glands) showed the greatest expression of ER-α, and also showed the most pronounced indication of basal cell squamous metaplasia in response to developmental exposure to low doses of DES and BPA relative to other regions of the prostate.

Conclusions

Because the control data of Tyl et al. (2008a) were not consistent with the prior published literature for prostate weight of young-adult CD-1 male mice and because their methods were inappropriate for revealing an extensive body of adverse effects detected using more sophisticated approaches, we deem the findings by Tyl et al. to be invalid. Hundreds of studies show adverse effects of BPA in animals, with many conducted at concentrations equivalent to current human levels of BPA exposure; thus, it is unlikely that academic scientists would bother to replicate the outdated approaches used by Tyl et al. (2008a, 2008b). This lack of replication is typical of GLP studies, which tend to involve unnecessarily large numbers of animals [Tyl et al. (2002) used > 8,000 rats], and reliability appears to be accepted because of the numbers of animals that were used. Although using excessive numbers of animals is accepted as good science by the U.S. FDA, the use of arbitrarily large numbers of animals per group (> 20 animals per treatment group is common) actually violates guidelines in the NIH *Guide for the*

Care and Use of Laboratory Animals (Institute of Laboratory Animal Research 1996) that govern research conducted by academic and government scientists. For research with animals to be approved by any university animal care and use committee, group sizes must be based on power analysis conducted using historic data. Based on this criterion in the NIH Guide, all of the studies by Tyl et al. were significantly over powered and thus in direct violation of federal guidelines for conducting animal research, a fact about which U.S. FDA regulators seem unaware.

Each of the four main industry-funded GLP studies of BPA (Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2008a, 2008b) is flawed and not appropriate for use in setting health standards. Clearly, meeting GLP standards is not a guarantee of reliable or valid science. It is of great concern that the U.S. and EU regulatory communities are willing to accept these industry-funded, antiquated, and flawed studies as proof of the safety of BPA while rejecting as invalid for regulatory purposes the findings from a very large number of academic and government investigators using 21st-century scientific approaches. The basis for these decisions by U.S. and EU regulatory agencies should be thoroughly investigated, particularly since the NTP (2008) concluded that BPA exposure to human infants was in the range shown to cause harm in experimental animals and since both the Canadian Ministry of Health and the Ministry of the Environment recently concluded that BPA was a toxic chemical (Environment Canada 2008).

Problems inherent with reliance on GLP as the standard for choosing data are compounded by the process used by federal agencies to determine membership on science advisory panels. Leading experts qualified by specific experience on the chemical or end points under consideration are often specifically excluded from membership. For example, the U.S. FDA's BPA review panel was identified as an expert panel, when in fact the panel was composed largely of scientists lacking any experience in research with BPA. This process, which appears to consider almost any scientist knowledgeable about a chemical to create bias, makes it vastly more difficult for the panel to integrate scientific data from the relevant literature, especially since, as with BPA, there are almost 1,000 relevant studies and the review panel is provided with very little time to become knowledgeable about the details. It means that the depth of knowledge present on this and similarly constituted government regulatory agency panels is unlikely to be sufficient to subject draft assessments to the scrutiny that peer review by experts normally entails. Combined with reliance on GLP data, this process has a high potential to yield flawed assessments that jeopardize public health.

We are not suggesting that GLP should be abandoned as a requirement for industry-funded studies. We object, however, to regulatory agencies implying that GLP indicates that industry-funded GLP research is somehow superior to NIH-funded studies that are not conducted using GLP. This argument demonstrates a lack of understanding of the profound difference between the use of replication as a mechanism to assess reliability and the methods used to assess validity for peer-reviewed published academic studies, whereas GLP was instituted with the expectation that this type of verification would not occur.

Public health decisions should be based on studies using appropriate protocols and the most sensitive assays. They should not be based on criteria that include or exclude data depending on whether or not the studies use GLP. Simply meeting GLP requirements is insufficient to guarantee scientific reliability and validity.

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総　説

Review

ダイオキシンによる免疫異常*

石丸直澄**　林　良夫**

Key Words：2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD), aryl hydrocarbon receptor(AhR), autoimmune disease, T cell

はじめに

環境ホルモンの一つとして知られるダイオキシン(図1)は免疫系，生殖系，神経系などの生物反応に重大な影響を及ぼすことが報告されてきた[1)~3)]．その中で免疫系への影響に関しては動物モデルを用いた研究が中心に行われ，免疫細胞の中で，T細胞やB細胞にダイオキシンの直接的な作用と間接的な影響に関して報告されてきた[4)~7)]．その中で，ダイオキシンのレセプターであるaryl hydrocarbon receptor(AhR)を介した分子シグナルの詳細が明らかにされようとしている．最近，ヘルパーT(Th)細胞の中でTh17細胞への分化をダイオキシンが調節することが判明した[8)]．さらに，Th17細胞が原因とされる自己免疫疾患の一つである多発性硬化症のモデルを用いた病態発症機序にダイオキシンが大きく影響を及ぼすことが明らかとされている[9)]．本稿ではこれまでのダイオキシンと免疫異常に関係する文献的知見を踏まえ，筆者らが明らかにしている自己免疫疾患に対するダイオキシンの影響に関する新知見を解説する．

免疫細胞へのダイオキシンの影響

正常マウスにダイオキシンを投与すると，胸腺が萎縮することが知られている[10)]．胸腺細胞の正負の選択に関連したアポトーシスにダイオキシンが影響を及ぼしている可能性や，胸腺間質細胞のFasLの発現にダイオキシンが調節因子として働きFasを発現した胸腺細胞のアポトーシスを制御しうるといったことが報告されているものの，明確な分子機序は不明である[11)]．さらに，ダイオキシン投与により，末梢のT細胞の機能低下が観察され，遅延型接触過敏反応やT細胞の細胞障害性活性の低下がみられる一方で，ダイオキシンによって各種刺激に対するT細胞の増殖反応やIL-2などのサイトカインの分泌は上昇することも知られている[12)13)]．また，卵白抗原(OVA)特異的なT細胞の反応性は初期の活性化には大きな影響は認められないかわりに，OVAに対するT細胞の増殖反応はダイオキシンによって亢進する[14)]．つまり，ダイオキシンの作用はT細胞の活性化ではなく生存に関係する分子群に影響を及ぼしている可能性がある．さらに，ダイオキシン投与により末梢での$CD25^+CD4^+$調節性T細胞(regulatory T cell；Treg cell)を誘導可能であるというユニークな報告もある[8)]．

一方で，ダイオキシンのB細胞への影響として，ヒツジ赤血球抗原の免疫に対する抗体産生はダイオキシンの投与により抑制され，さらに，lipopolysaccaride(LPS)あるいはIgM抗体などによる刺激でB細胞の増殖反応がダイオキシン添加により阻害されることも報告されている[15)]．B細胞の最終分化段階である形質細胞への分化をダイオキシンが阻害する結果も知られている[16)]．加えて，ダイオキシン投与マウスへのインフル

* Immune disorder by dioxin.

** Naozumi ISHIMARU, D.D.S., Ph.D. & Yoshio HAYASHI, D.D.S., Ph.D.: 徳島大学大学院ヘルスバイオサイエンス研究部口腔分子病態学分野〔〒770-8504 徳島市蔵本町3-18-15〕; Department of Oral Molecular Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8504, JAPAN

エンザウイルスの感染実験では，T 細胞，B 細胞の機能低下とともにインフルエンザウイルスに対する抗体の産生も劇的に抑制されることが判明した[17]．

また，LPSの腹腔内誘導によるマクロファージの活性化をダイオキシンがTNF-αの産生上昇を介して亢進させる働きがある[18]．さらに，樹状細胞へのダイオキシンの影響については，抗原の取り込みや活性化に関してはダイオキシンが阻害的効果を有しているものの，T 細胞への抗原提示能を上昇させる作用があることも報告されている[19]．

ダイオキシンは免疫細胞の種類やそれらの細胞の種々の機能に対して幅広い影響が認められるが，免疫細胞の機能に対して抑制的な効果が目立つ．表 1 にそれぞれの免疫細胞におけるダイオキシンの影響についてまとめる．

ダイオキシンによる細胞内分子シグナル

細胞内に入ったダイオキシンは細胞質に存在するそのレセプターであるAhRと結合する(図2)．AhRはヘリックス-ループ-ヘリックス(helix-loop-helix；HLH)ファミリーに属する転写因子として知られている．ダイオキシンと結合して活性化したAhRはAhR nuclear translocator(ARNT)とヘテロダイマーを形成し，核内に移行した後，さまざまな遺伝子上に存在するdioxin responsive element(DRE)として知られるxenobiotic response element(XRE)に結合することによりその遺伝子の転写が調節される[1)20)21)]．AhR複合体の標的遺伝子として，もっとも知られているのがcytochrome P-450 1A1(CYP1A1)である．CYP1A1は増殖・アポトーシスなどの細胞の生死を中心

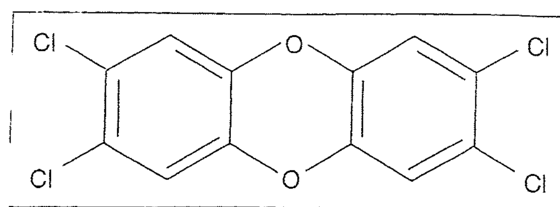

図 1　ダイオキシン〔2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD)〕の化学式

に重要な役割を果たしており，AhRを介したダイオキシの細胞内における分子機序を解析するのに有効なメルクマールとして広く知られている[22)～24)]．また，AhRはnuclear factor-κB(NF-κB)のサブユニットの一つであるRelBとも結合することにより，免疫反応に重要な転写因子であるNF-κBの制御に影響を及ぼしていることも報告されている[25]．AhRを中心としたダイオキシンの分子シグナルの解析にはAhRノックアウトマウスを用いることで明確な現象を観察することが可能となる．また，AhRノックアウトマウスを用いた免疫細胞への実験に関しても機能解析を中心に多くの報告がなされてきた[1)10)26)]．

ダイオキシンは，AhRを起点として，CYP1A1やNF-κBを介した多彩な免疫細胞機能に対して複雑に影響を及ぼしている．しかし，AhRのリガンドはダイオキシンだけでなくさまざまな生体物質あるいは非生体物質があげられることに加えて，ダイオキシン自体が内分泌かく乱物質としてエストロジェンレセプターと相互作用することにより，本来性ホルモンで制御されている生体機能のホメオスターシスの維持を破綻させる複雑な分子機序を有していることから，実際の生体内で起こっているダイオキシンの詳細な動態，正確な分子シグナルに関しては多くの謎が残されている．

表 1　免疫細胞におけるダイオキシンの影響

免疫細胞	TCDDによる影響	文献番号
胸腺細胞	アポトーシス亢進	10)11)
T 細胞	細胞障害性低下，増殖反応低下，Th17分化	8)9)12)～14)
調節性 T 細胞	誘導	8)
B 細胞	抗体産生低下，増殖反応低下	15)～17)
マクロファージ	活性化亢進	18)
樹状細胞	活性化低下，抗原提示能亢進	19)

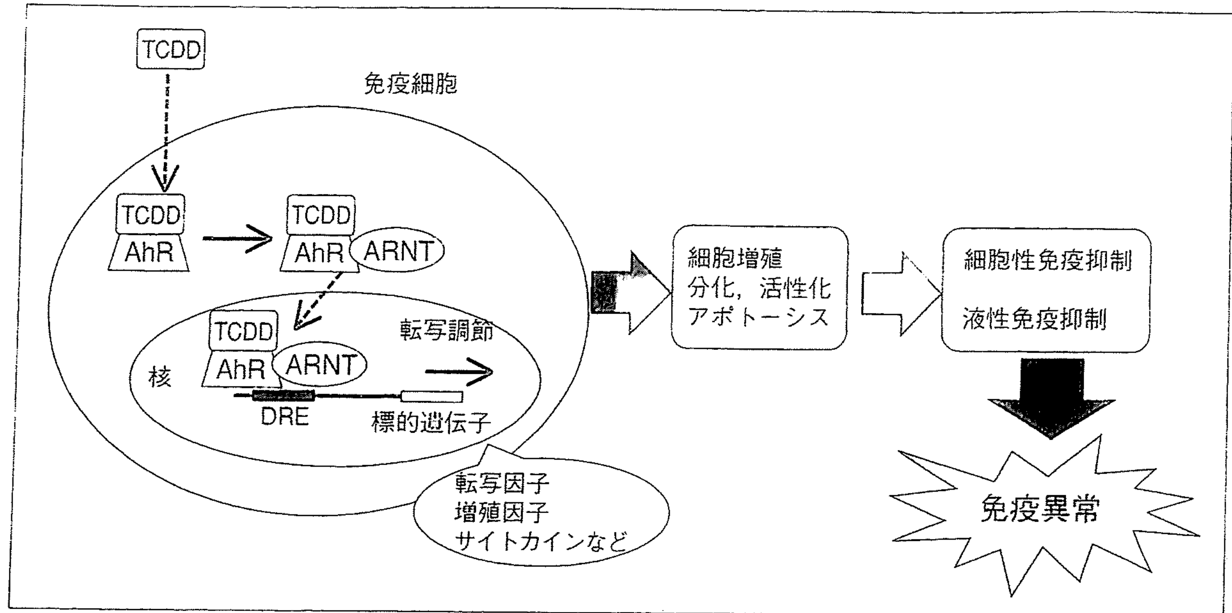

図２　免疫細胞におけるAhRを中心とした分子シグナル

ダイオキシンとTh17細胞

最近，Nature誌の同号にAhRとＴ細胞分化あるいは自己免疫疾患との関係を決定づける２本の論文が発表された[8)9)]．ダイオキシン（TCDD）の刺激で活性化されたAhRを介してTreg細胞においてもっとも重要とされる転写因子Foxp3の発現が亢進されることによって，Treg細胞の細胞数が増加することが判明した．さらに，多発性硬化症のモデルマウス〔実験的自己免疫性脳脊髄炎（experimental autoimmune encephalomyelitis；EAE）〕にダイオキシンを投与することによりTreg細胞の増加を介する病態の抑制効果があることを見出した．しかしながら，AhRの内因性リガンドの一つである6-formylindolo[3,2-b]carbazole（FICZ）をEAEモデルマウスに投与すると病態は増悪した．FICZはＴ細胞のIL-22およびIL-17の産生を上昇させることにより，EAEの病態形成にきわめて重要なTh17細胞の分化を促進させていることが明らかとなった．TCDDの投与で増加していたTreg細胞に関してはFICZ投与では影響がなかった．さらに，AhRの別のリガンドであるβ-naphthoflavoneを用いた実験においても，FICZと同様の効果が認められた．AhRは複数のリガンドと結合するため，リガンド依存性の転写制御機構が存在するものと考えられている．Th17細胞は従来知られていたIFN-γやIL-2などのサイトカインを分泌するTh1細胞とIL-4やIL-10などを分泌するTh2細胞とは異なるＴ細胞セブセットとして同定され，多発性硬化症などの自己免疫疾患の病態発症に重要な役割を果たしているという多くの報告がなされている．Nature誌に報告された２本の論文では共通してFICZは健常人の皮膚に存在し，紫外線によって活性型となりAhRと結合することが知られている．ダイオキシンや他のリガンドとAhRとの結合様式や親和性などいくつかの相違点があるものの，AhRの活性化機構に関しては不明な点が多い．また，Treg細胞におけるダイオキシンによるFoxp3の発現亢進の分子機序に関しても議論の余地を残している．

ダイオキシンと自己免疫疾患

上述のEAEの発症にFICZの投与によってAhRを介したＴ細胞異常に起因した自己免疫疾患の悪化効果があることが判明したものの，ダイオキシンが自己免疫疾患に影響するか否かは不明のままである．筆者らはこれまでに，唾液腺，涙腺を標的臓器とする自己免疫疾患であるシェーグレン症候群（Sjögren's syndrome；SS）のモデルマウスを確立し，その病態に関し研究を進めてきた[27)~29)]．SSの臨床病態は閉経期以降の女性に発症ピークを有し，ドライアイ，ドライマウスなどの乾燥症候群を呈し，血清自己抗体として抗SSAあるいは抗SSB抗体が検出され，小唾液腺の口唇生検により導管周囲性のリンパ球浸潤が

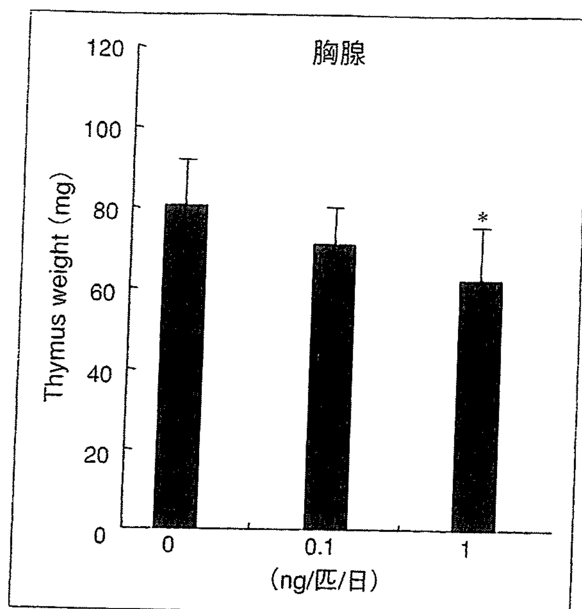

図3　新生仔期にTCDDを投与した2か月齢での胸腺の臓器重量

観察される[30]．筆者らが確立したSSのモデルマウスは舌下腺の分化異常をきたすことが知られているNFS/*sld*マウスに生後3日目においてT細胞の教育の場所である胸腺を外科的に切除することによって若齢期から高率に唾液腺，涙腺に限局する自己免疫性病変が観察される[27]．ダイオキシンによって胸腺細胞のアポトーシスが亢進するという報告に着目して，本マウスの新生仔期に胸腺を摘出する代わりにダイオキシンを投与することによって自己免疫病変が誘導されるか否かを検討した．ダイオキシンを投与されたマウスでは2か月齢において胸腺の臓器重量が対照群に比較して有意に減少していた(図3)．ダイオキシン投与により唾液腺には2か月齢より本来のモデルマウスで観察される自己免疫病変に類似した炎症性病変が観察された(図4)．病態誘導の詳細な分子機構に関しては不明であるが，新生仔期にダイオキシンに曝露されることにより胸腺の分化や成熟に異常が発生し，自己，非自己を区別する中枢性免疫寛容システムが破綻することにより，自己免疫疾患が発症したものと想定される．このことはヒトの新生児期や若齢期にダイオキシンが仮に曝露されたとすると，将来的に自己免疫疾患の発症リスクが上昇してしまう可能性を示唆している．しかし，自己免疫疾患は一つの因子で発症が決定づけられるわけではなく，遺伝因子や環境因子などが複雑に絡み合って中枢性および末梢性免疫寛容の破綻に結びついていくものと理解されているので，ダイオキシンそのものが自己免疫疾患の発症を直接的に左右しているとは言いがたい．そのレセプターであるAhRを起点とした分子シグナルの複雑さを考慮すると，ダイオキシンによる自己免疫疾患の発症に及ぼす影響には，病態に関与する免疫細胞および標的臓器細胞などへのAhRを介した分子機序に内在性のAhRリガンド，さらにホルモンなどのダイオキシンとの相互作用などさまざまな因子を考慮する必要がある．

おわりに

ダイオキシンの生体への影響に関してはその濃度が重要であることが知られている．動物実験では比較的高濃度での研究が進められているが，低濃度のダイオキシン曝露により晩発性の影響(low dose late effect)がすでに知られている．発癌，免疫異常，代謝異常など年齢という因子によって発症がある程度左右される疾患に関し

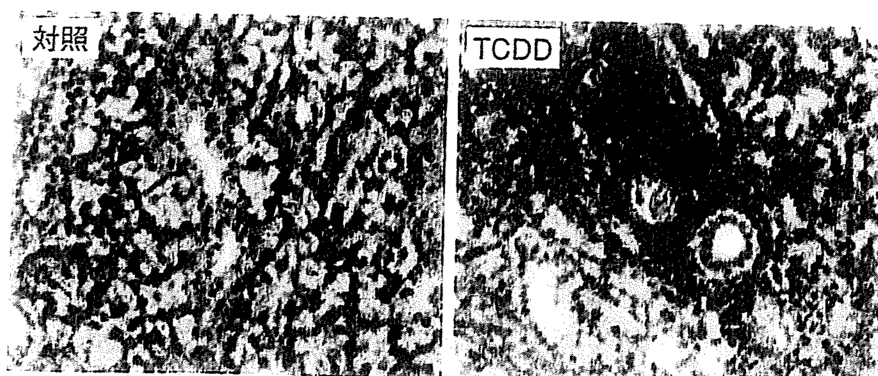

図4　新生仔期にTCDDを投与したマウスの唾液腺組織(Hematoxylin & Eosin染色)

てはダイオキシンの晩発性の影響は小さくないものと考えられる．たとえば，幼少期にダイオキシンに低濃度で曝露される環境にあれば，免疫疾患の好発年齢でより発症するリスクは高くなるのかもしれない．内分泌かく乱物質はダイオキシンだけではなく，われわれが生活する中で数多くの物質が生体内に入ってくる可能性があり，その中で内分泌かく乱物質として生体の恒常性を破綻してしまうものも現在知られているもの以外に存在する恐れもある．十年以上前に動植物のメス化とダイオキシンを代表とする内分泌かく乱物質の関係がクローズアップされてから，さまざまな角度から明らかにされてきたダイオキシンの分子メカニズムに関する研究は今後起こりうる人類に向けられた予言的な警告であると考えられる．生体システムにおいていまだ全容解明にまで至っていないさまざまな化学物質による“かく乱”の分子機構が今後明らかにされる必要がある．

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