Modulation of Nuclear Receptor Cofactor Recruitment by Tributyltin and Dibutyltin in Gal4 Assays

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Introduction

Widespread use of organotin compounds in agriculture and industry has caused increasing amounts to be released into the environment. Trisubstituted organotin compounds have biocidal properties and are used in agriculture as fungicides and acaricides, and as rodent repellents and mulluscicides, and they are widely used as antifoulants in ship paints and underwater coatings, especially, triphenyltins (TPTs) and tributyltins (TBTs) have been used extensively in antifouling products as algaecides and mulluscicides. Disubstituted organotin compounds are commercially the most important derivatives, and are mainly used in the plastics industry, particularly as heat and light stabilizers. Moreover, in environment, tributyltin (TBT) is degradaded spontaneously and biochemically via a debutylation pathway to dibutyltin[1, 2]. TBT causes the reproductive toxic effects to marine gastropods, which effects were represented by some masculinizing effects including "imposex" or "psuedohermaphoditism"[3]. Dibutyltin (DBT) dose not induce imposex in gastropods species [4].

Recently it was elucidated that tributyltins bound to nuclear receptors, such as RXR, PPARγ, and activated RXR-dependent signaling pathways[5-7],while DBT does not bind to RXR, nor does it activate the pathways[7]. Such kind of nuclear receptor dependent mechanism appear to contribute the induction of hCG secretion and aromatase activity in human placenta-derived cells[7], as well as the masculinizing effects in gastropods[5].

We summarized the data of the studies on reproductive and developmental toxicity of organotins in mammals[8]. Both DBT and TBT caused reproductive and developmental adverse effects in experimental animals. Dibutyltin dichloride (DBTCl) during early pregnancy produced pregnancy failure in rats[9, 10], as well as tributyltin chloride (TBTCl)[11-13]. Predominant adverse effects of DBTCl on days 0-3 of pregnancy were decrease in the pregnancy rate and increase in the incidence of pre-implantation embryonic loss. The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl[10]. Our study recently suggested that the decline in progesterone levels is primary mechanism for the implantation failure due to DBTCl[14]. The molecular mechanism of decrease of progesterone levels remains unclear. The failure of implantation may be involved in mechanisms other than activation of RXRs. To seek the molecular targets of the implantation failure by organotins, we analyzed modulation of nuclear receptor cofactor recruitment by tributyltin and dibutyltin in Gal4 assays.

Materials and Methods

The compounds Tributyltin (Sigma Aldrich 45713) and Dibutyltin (Sigma Aldrich 34920) were tested in Phenex Nuclear Receptor Gal4 transactivation assays. All transient transfections were done in HEK293 cells (obtained from DSMZ (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany). For transfection, $5x10^5$ cells were plated per well in 96well plates in minimum essential medium (Eagle) without Phenol Red and L-Glutamine and with Earle's BSS supplemented with 10% charcoal/dextran treated FBS (HyClone, South Logan Utah). These cells were transiently transfected the following day at >90% confluence using a polyethylene-imine-based transfection-reagent (Polyethyleneimine, Aldrich Cat No. 40,827-7). Compound stocks were prepared in DMSO, prediluted in medium and added 2-4 hours after addition of transfection mixture (final vehicle concentration not exceeding 0.1%). Cells were incubated for additional 16 hours before firefly and renilla luciferase activities were measured sequentially in the same cell extract using a Dual-Light-Luciferase-Assay system[15].

The plasmids used in Phenex' Gal4 assay system are derivatives of Stratagene's M2H plasmids: the reporter plasmid pFR-Luc (contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control expression of the *Photinus pyralis* (American firefly) luciferase gene), and pCMV-BD (for fusions of Nuclear Receptor ligand binding domains to the DNA-binding domain of the yeast protein GAL4). In case of AR and ERRalpha we did a M1H assay with the AR (ERRalpha) LBD fused to the pCMV-BD vector and added PGC1, as a pTRex construct, as this is a possibility to enhance the assay window, still allowing activity modulation by interacting compounds, such as DHT and DES.

In order to improve experimental accuracy a second reporter - *Renilla reniformis* luciferase, driven by a constitutive promoter - was included as internal control[16]. Using the control reporter (Renilla Luciferase) corrects for variations in experimental handling e.g. transfection efficacy, cell viability, pipetting errors, cell lysis efficiency and assay efficiency. In our hands it definitely is an additional indicator for transfection efficiency and comparability of experiments.

Results and Discussion

Human GR, MR, PR, PPARα, PPARβ/δ, PPARγ, RXRα, RXRβ, RXRγ, RARα, LXRα, FXR, VDR, ERRα, AR, PXR, CAR and the non human receptors mouse and rat PPARγ, rat RARα, rat RARβ and rat RARγ were tested in agonist mode. Both organotins were incubated with a maximum concentration of 150nM in all assays, as higher concentrations seriously reduced vitality of the cells.

DBT partially activates human, murine and rat PPARγ with an EC_{50} of about 60nM (Fig.1) and has a very weak agonistic effect on human PXR. DBT is shown to exhibit a weak antagonistic effect at ERRα at very low concentrations (IC_{50}: ~0.25nM). DBT shows a signal decrease in the human PPARα assay and the three rat RAR assays. TBT fully activates the three human RXRs with EC_{50} values of 15-20nM (result not shown). Similar to DBT, TBT partially activates human, murine and rat PPARgamma with an EC_{50} of about 40nM. TBT also weakly activates PPARβ/δ and partially antagonizes ERRα at very low concentrations (IC_{50}: ~0.5nM). TBT shows a signal decrease in the AR assay and the three rat RAR assays.

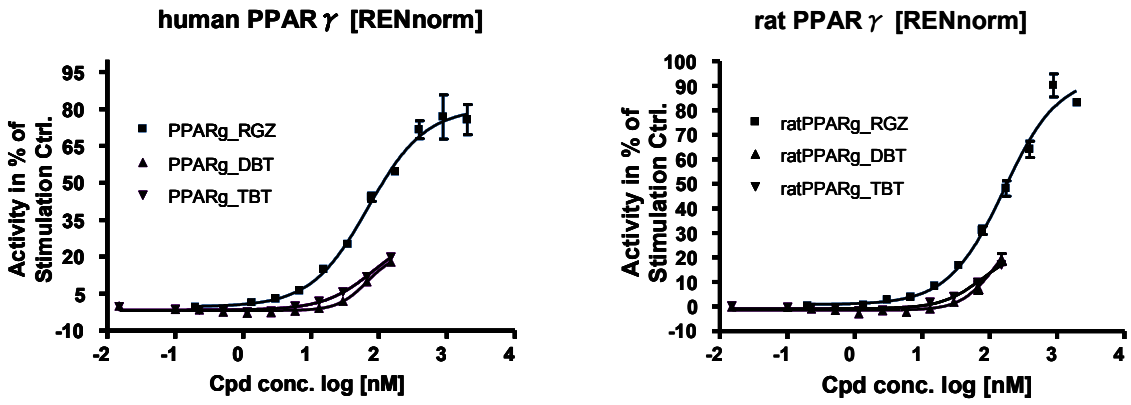

Fig. 1. The partial PPARγ activation by DBT ad TBT in agonist mode assays
(RGZ: control ligand of rosiglitazone.)

In order to confirm the antagonistic activity, we performed the GAL4 assays for PR, PPARα, PPARγ, AR, rat RARα, rat RARβ and rat RARγ in antagonist mode, when the nuclear receptor is already activated with sub-saturating concentrations of reference compound. TBT shows an antagonistic effect (40-50% of the maximum signal) in PR antagonist assays, which has been induced by sub-saturating reference agonist concentrations. In PPARα antagonist assay, DBT and TBT show antagonistic effects at concentrations below 5.9nM. In PPARγ antagonist assay, TBT shows very potent (IC_{50}: 0.2-0.4nM) partial antagonistic activity (~40%

of the maximum signal). Also DBT causes a signal decrease at lower concentrations, but causes an increase at the highest concentrations (Fig.2). As this increase in high concentration is observed in all performed antagonist mode assays using DBT, we regard this as an unspecific effect. In the antagonist mode AR assay, TBT shows clear antagonistic activity (IC_{50}: 30-50 nM), reducing the signal to 30-40% of the maximum signal, which has been induced by sub-saturating reference agonist concentrations (Fig 3).

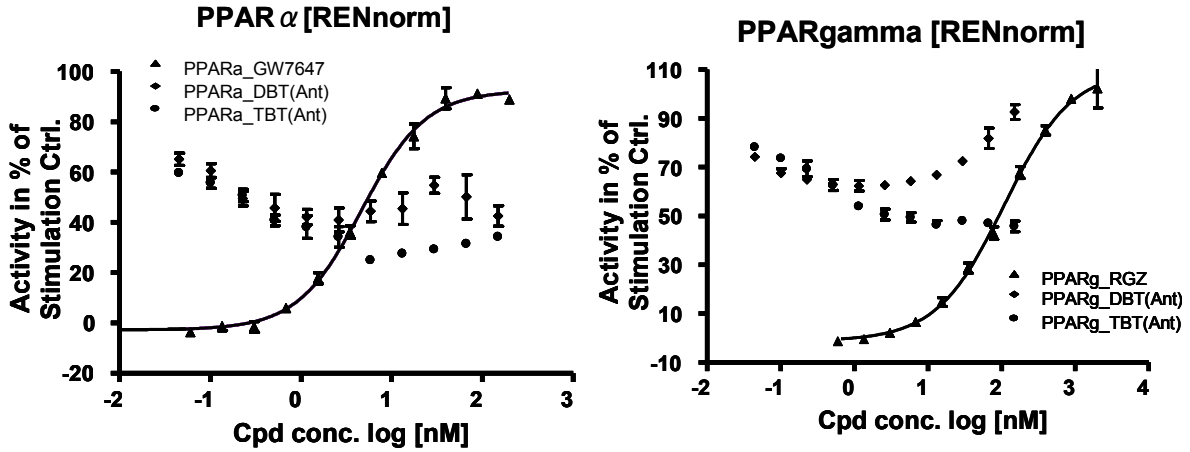

Fig. 2. The partial antagonistic activities by DBT ad TBT in antagonist mode assays.
(GW7647: PPARα agonist; DHT: dihydroteststerone.)

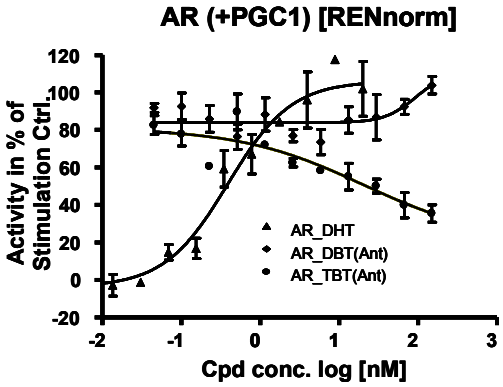

Fig. 3. The AR antagonistic activities by TBT in antagonist mode assays.
(DHT: dihydroteststerone.)

There have been previous reports of the PPARγ-RXR agonistic effects by TBT[5-7], which we cofirmed in our Gal4 assay system. Although DBT could not activate the RXR, similar levels of partial activations by either DBT or TBT were observed in human, murine and rat PPARγ. The ranges of EC_{50} in the partial activation of PPARγ by both compounds are around 50nM, and are higher than EC_{50} of RXR activation (15-20nM) by TBT. The activation pathway of PPARγ in these concentration ranges would not contribute to the failure of implantation, which was caused by either TBT or DBT. However, the partial PPARα and PPARγ antagonistic effects by TBT and DBT in antagonist assays were observed at lower concentrations (PPARα: below 5.9nM; PPARγ: below 0.4nM) than the RXR activated concentration. It is well known that PPARγ is required for placenta development[17, 18]. Also it was reported that PPARγ activation and gene expression of CYP11A and CYP17 have linked to the progesterone production in porcine theca cells[19]. We recently observed the decreased gene expression of CYP17 in mice ovary by DBT treatment during implantationin[20]. Taken together, the partial antagonistic effects of PPARγ in these lower concentrations might have contributed to the failure of implantation,

which has been observed in mammals by treatment with either DBT or TBT. Additionally we found clear AR antagonistic activity by only TBT. The activity might be involved in unidentified mechanisms associated with TBT toxicity.

Acknowledgements
This work was supported by Health and Labour Sciences Research Grants (Research on Community Health Crisis Management; H19-Kenki-Ippan-012) from the Ministry of Health, Labour and Welfare of Japan.

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