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Endocrine Disrupting Organotin Compounds are Potent Inducers of Imposex in Gastropods and Adipogenesis in Vertebrates

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Abstract

The persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces not only imposex in gastropods but also the differentiation of adipocytes *in vitro* and increases adipose mass *in vivo* in vertebrates. TBT is a nanomolar affinity ligand for retinoid X receptor (RXR) in the rock shell (*Thais clavigera*) and for both the RXR and the peroxisome proliferator activated receptor γ (PPARγ) in the amphibian (*Xenopus laevis*), mouse, and human. The molecular mechanisms underlying induction of imposex by TBT have not been clarified, though several hypotheses are proposed. TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways *in vivo* primarily through activation of RXR and PPARγ. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased adipose mass in adults. In *X. laevis*, ectopic adipocytes form in and around gonadal tissues following organotin, RXR or PPARγ ligand exposure. TBT represents the first example of an environmental endocrine disrupter that promotes adverse effects from gastropods to mammals.

Keywords: TBT, Gastropods, *Xenopus laevis*, Mouse, Human, RXR, PPARγ, Imposex, Adipogenesis

Organotins are a diverse group of widely distributed environmental pollutants. Tributyltin chloride (TBT) and bis (triphenyltin) oxide (TPT), have pleiotropic adverse effects on both invertebrate and vertebrate endocrine systems. Organotins were first used in the mid-1960s as antifouling agents in marine shipping paints, although such use has been strictly restricted in recent years. Organotins persist as prevalent contaminants in dietary sources, such as fish and shellfish, and through pesticide use on high value food crops[1,2]. Additional human exposure to organotins may occur through their use as antifungal agents in wood treatments, industrial water systems and textiles. Mono- and di-organotins are widely used as stabilizers in the manufacture of polyolefin plastics (polyvinyl chloride), which introduces the potential for transfer by contact with drinking water and foods.

Exposure to organotins such as TBT and TPT results in imposex, the superimposition of male sex characteristics in (or "on") female gastropod mollusks[3-5]. Imposex results in impaired reproductive fitness or sterility in severely affected animals and TBT exposure represents one of the most clear cut examples of environmental endocrine disruption. TBT exposure also leads to masculinization of at least two fish species[6,7]. In contrast, TBT exposure results in slight effects on the mammalian reproductive tracts and has not been reported to alter sex ratios[8,9]. Hepatic-, neuro- and immunotoxicity are reported to be the major effects of organotin exposure in mammals[10]. Our current understanding of how organotins disrupt the endocrine system is based on how organotins affect the expression or activity of steroid regulatory enzymes such as P450 aromatase together with less specific toxic effects resulting from damage to mitochondria and immune cells[11-15]. The currently available data do not permit one to draw firm conclusions regarding whether organotins function primarily as protein and enzyme inhibitors *in*

vivo, or instead regulate gene expression in a more direct manner. In this review, current possible hypotheses of imposex induction by organotin compounds and newly identified molecular mechanism of adipogenesis induced by organotins are summarized.

Possible Mechanisms in Induction of Imposex by Organotins in Snails

Approximately 150 gastropod species worldwide (including the rock shell) and 39 gastropod species in Japan, were reported to show imposex caused by organotins[5,16,17]. Gastropod imposex was induced by very low concentrations of TBT and/or TPT[18-26]. In severely affected stages of imposex, reproductive failure resulting from either oviduct blockage by vas deferens formation or ovarian spermatogenesis leads to population declines and mass extinction[4,5,22,27]. Imposex in gastropods is also thought to be a clear manifestation of endocrine disruption in wildlife[27].

To date, five possible mechanisms concerning imposex induction in gastropods have been proposed: 1) increased androgen levels caused by aromatase inhibition by TBT in the dog-whelk (*Nucella lapillus*)[28-30]; 2) inhibition of the excretion of sulfate conjugates of androgens by TBT in *Littorina littorea*[31]; 3) disturbance of the release of penis morphogenetic/retrogressive factor from pedal/cerebropleural ganglia by TBT in the sting winkle (*Ocenebra erinacea*)[32]; 4) increase in the neuropeptide APGWamide level caused by TBT in the mud snail (*Ilyanassa obsoleta*)[33,34]; 5) activation of retinoid 'X' receptor (RXR) by TBT induced penis morphogenesis and promoted the development in the rock shell (*T. clavigera*)[35]. These studies were conducted using different species of gastropods; therefore, it may not be possible to derive a common mechanism for the induction of imposex.

Effects of Androgen and Aromatase Inhibitor on the Imposex Development

The proposed mechanism of imposex induction by organotins has been critically reviewed by Horiguchi[17]. No clear correlation has been demonstrated among aromatase inhibition, androgen increase and penis growth to support the aromatase inhibition hypothesis. There is no evidence supporting the presence of either the aromatase gene or the androgen receptor (AR) in gastropods, further calling this model into question. Perhaps the most decisive evidence comes from our studies, which showed that direct injection of androgen into female rock shells did not induce imposex.

We injected live rock shells (*T. clavigera*) collected at Hiraiso (known as a less polluted site by TBT and TPT), Japan with the aromatase ($P450_{Arom}$) inhibitor fadrozole (Fad) and testosterone (T) as well as a positive control triphenyltin chloride (TPTCl). After 1 month, the animals were examined for imposex[24]. The positive control, TPTCl, clearly promoted the development of imposex (Table 1). Fad seemed to have significantly affected penis length, but the penis length was very small and the variance was large. No significant effects of Fad on the development of imposex or the VDS index in the rock shell were observed (Table 1). No significant effect was noted for the combined effect of Fad and T for the development of imposex in the rock shell, based on the incidence of imposex, penis length and VDS index after a month of injections (Table 1). Moreover, Fad (0.3 mg/L) and Fad (0.3 mg/L)+T (0.1, 1, and 10 μg/L) in 3-month flow-through exposure experiments did not significantly promote imposex symptoms in the rock shell (Sugimoto, Horiguchi, Shiraishi, Morita, Takahashi & Miura, unpublished data). Taken together, these results suggest that any expected increase in androgen levels due to inhibition of a postulated $P450_{Arom}$ cannot be a primary factor in the development of gastropod imposex.

The androgen excretion hypothesis is also based on the idea that penis is induced by androgen. However, there is no clear evidence showing androgen induction of penis in gastropods. The penis morphogenetic hypothesis is based on results showing toxic effects of organotins on isolated nervous systems of penis-forming area. No clear evidence of the penis morpho-

Table 1. Imposex incidence, penis length, and vas deferens sequence (VDS) index (mean ± standard deviation) in female rock shells 1 month after injection.

	Ethanol (negative control)	Fadrozole	Fadrozole + testosterone	TPTCl
Incidence (%)	15	35	20	100**
Penis length (mm)	0.02 ± 0.10	0.12 ± 0.19*	0	3.10 ± 3.57***
VDS index	0.20 ± 0.52	0.70 ± 1.03	0.20 ± 0.41	2.80 ± 1.51***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (χ^2 analysis for the incidence of imposex, and ANOVA for penis length and VDS index)
The amount of injected test solution was calculated, based on estimated (nominal) tissue concentrations, as 5, 0.1, and 1 μg/g wet tissue for Fad, T, and TPTCl, respectively. After injection of the test solution (n=20 females per group; once or twice injections per solution), each group was kept in a 2-L glass beaker with flow-through artificial seawater (10 L/day) and live mussels as food

genetic factor has been demonstrated. Moreover, the reported penis inducing activity of the APGWamide neuropeptide was not strong enough to support this hypothesis[17].

In contrast to the studies regarding androgen synthesis, both TBT and TPT activated the rock shell RXR *in vitro* and natural ligand of RXR, 9-*cis* retinoic acid (RA) induced the development of imposex (penis formation and growth) in the female rock shells *in vivo*, supporting the RXR hypothesis. To date, this is the most realistic hypothesis for the molecular mechanism of imposex induced by organotins. However, detailed experiments will be needed in the future to provide *in vivo* evidence linking RXR activation to penis formation. It will also be necessary to demonstrate which pathways downstream of RXR are required for penis morphogenesis. It should be noted that RXR is required as an obligate heterodimeric partner for other nuclear receptors, both in vertebrates and invertebrates. Therefore, one cannot currently exclude the possibility that imposex might be induced through one or more RXR partners.

Testosterone is Converted to 17β-estradiol in Rock Shell Gonad Extracts

The model that TBT inhibits aromatase to increase T while decreasing 17β-estradiol (E2) requires the presence of an aromatase activity. Since no convincing evidence was available regarding aromatase in the rock shell, we undertook to measure the conversion of testosterone to E2 in the rock shell gonad/digestive gland complex extracts. We found that the gonad extract was able to convert a small amount of testosterone to E2 (Figure 1). This result provides biochemical evidence that the rock shell gonad/digestive gland complex contains a functional aromatase activity that catalyzes production of estrogen from testosterone *in vitro*. However, it should be noted that the production of E2 was relatively low level, compared with that catalyzed by the cloned aromatase from Nile Tilapia (*Oreochromis niloticus*)[36]. This raises the possibility that the rock shell activity may not be a bona fide aromatase, but rather a secondary activity of another enzyme. Further studies, including the cloning of rock shell aromatase and a detailed examination of rock shell steroid levels will be required to clarify this issue.

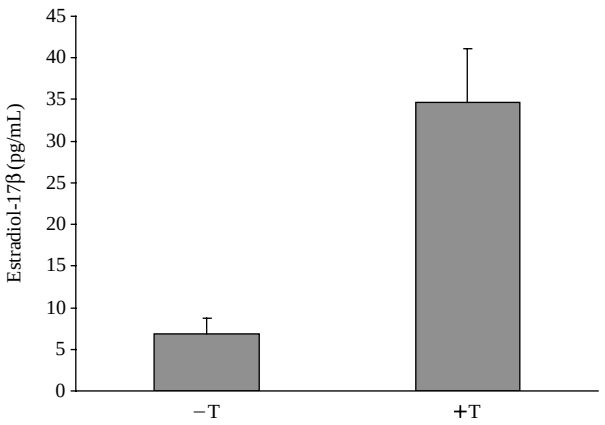


Figure 1. Testosterone (T) is converted to 17β-estradiol (E2) in the extract of the rock shell gonad/digestive gland complex. The rock shell gonad/digestive gland complex were extracted and incubated with T. After incubation, the samples were extracted twice with diethyl ether and evaporated by vacuum centrifugation. E2 in this extract was measured with an Estradiol-17β Enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

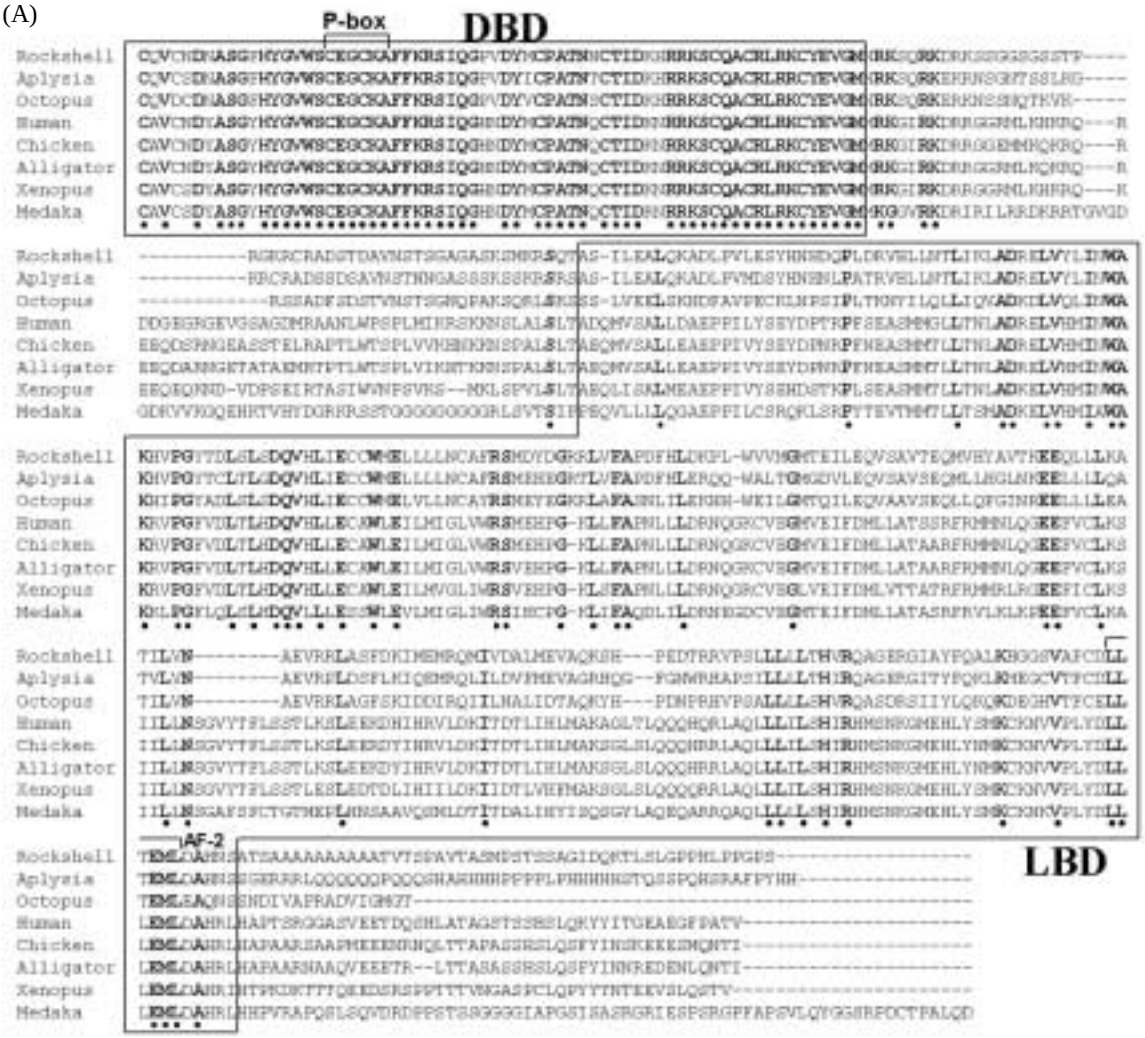
Rock Shell ER-like Protein cDNA

Recently, Kajiwara *et al*.[37] cloned a gene encoding a rock shell estrogen receptor (ER)-like protein. We independently cloned an ER-like sequence from the rock shell (Figure 2). Using the nomenclature of Krust *et al*.[38], the rock shell ER-like protein sequence can be divided into five domains, the N-terminal region, DNA binding domain (DBD), hinge, ligand binding domain (LBD) and C-terminal extension, based on its sequence similarity to other steroid hormone receptors. In the DBD, the rock shell ER-like protein has the highest protein sequence identity to the aplysia (*Aplysia californica*) ER (93%), octopus (*Octopus vulgaris*) ER (93%), human ER (89%), chicken ER (89%), alligator (*Alligator mississippiensis*) ER (89%), *Xenopus* ER (87%) and medaka (*Oryzias latipes*) ER (86%), and much lower similarity to other steroid receptors, human AR (59%), human progesterone receptor (56%), human glucocorticoid receptor (57%) and human mineralocorticoid receptor (57%). In addition, in the P-box of the DBD, a highly conserved motif for DNA recognition[39,40], the rock shell protein has the signature sequence of ER, but not other receptors (Figure 2).

Transactivation of the Rock Shell ER-like Protein

We employed several molecular assays to determine the function of the rock shell ER-like protein. Since the sea hare ER and octopus ER are ligand-independent for transactivation in reporter gene assays, we first tested whether the transactivation of the rock shell ER is ligand-depend or ligand-independent. We prepared a fusion construct of the GAL4-DBD with the rock shell ER-like protein, including C, D, E and F domains, and expressed it in CHO-K1

(A)



(B)

	P-box	DBD (%)	LBD (%)
rockshell	CEGCKA	-	-
aplysia	CEGCKA	93	74
octopus	CEGCKA	93	60
human ER	CEGCKA	89	37
human ERR	CEACKA	68	31
human AR	CGSCKV	59	26
human PR	CGSCKV	56	26
human GR	CGSCKV	57	25
humnan MR	CGSCKV	57	25

Figure 2. Amino acid sequence of the rock shell estrogen receptor (ER)-like protein (rER). (A) The rER and ERs of other animals were aligned with CLUSTAL W software. The DNA binding domain (DBD) and ligand binding domain (LBD) are enclosed within a box. The P-box and AF-2 region are indicated with lines. The closed circles indicate residues conserved in all ER members. (B) The similarity of the rER and the ERs of other species. The amino acid sequences of the P-box in the DBD of the rER are compared with other steroid hormone receptors. The percent of the alignment amino acid that is identical between the rER and other steroid hormone receptors in the DBD and LBD.

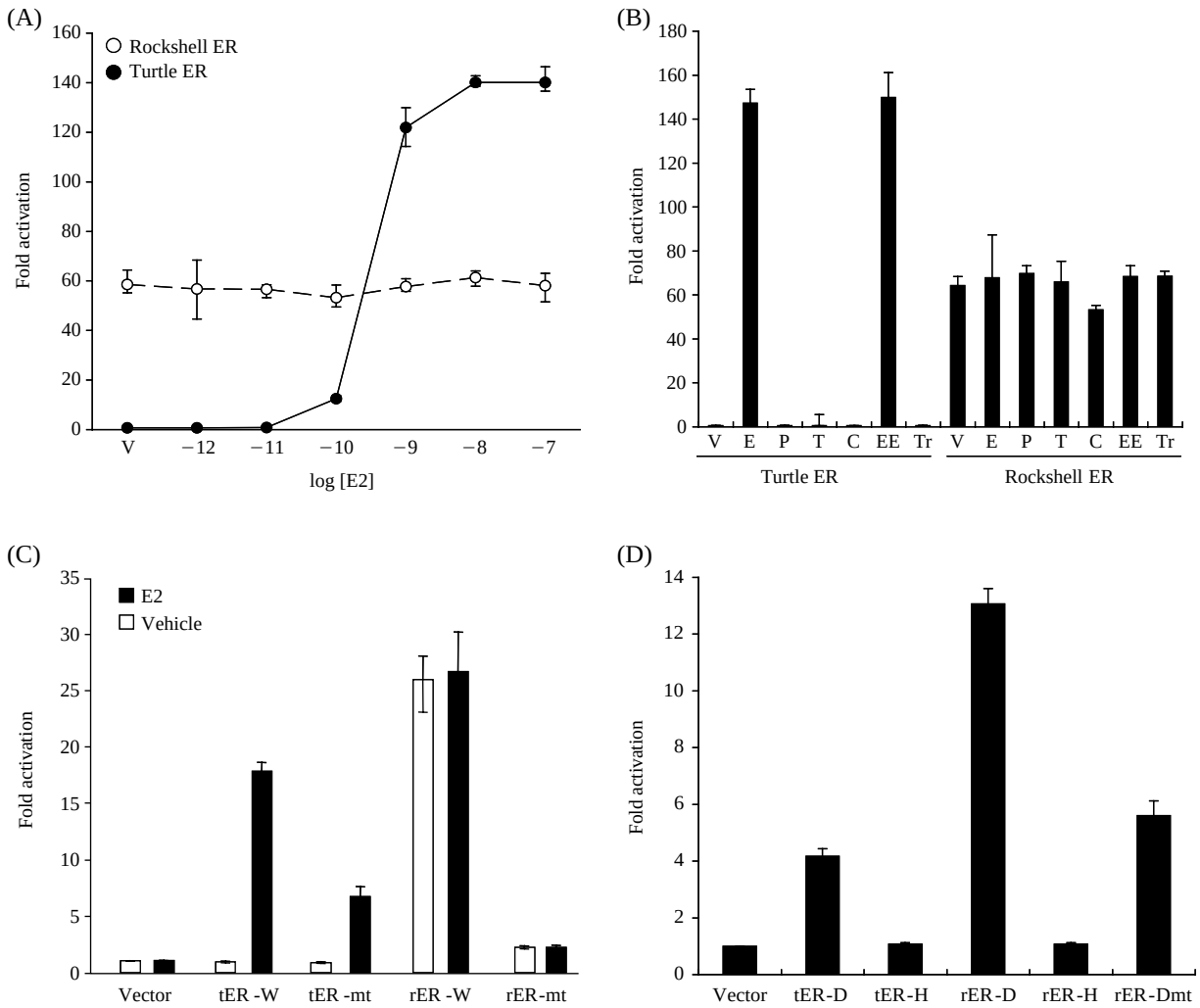


Figure 3. Transcriptional activities of the rock shell ER-like protein (rER). For A and B, the rER or freshwater turtle ER (tER) were expressed in CHO-K1 cells as fusion proteins with a GAL4-DBD. The fold activation indicates luciferase activity relative to the vector control containing GAL4-DBD. Mean ± SE of 3 replicates is shown. (A) The rER is constitutively active and does not respond to increasing concentrations of E2. Cells were treated with vehicle (DMSO) or with E2. (B) The rER does not respond to steroid hormones. Cells were treated with 10 nM of various hormones or with vehicle (DMSO). E, estradiol-17β; P, progesterone; T, testosterone; C, cortisosterone; EE, ethinylestradiol; Tr, Trenbolone. (C) AF-2 mutation of the rER abolished the transcriptional activity. Wild-type and mutated in AF-2 region of rER and tER were expressed in CHO-K1 cells. Cells were treated with 10 nM E2 or with vehicle only (DMSO). Each bar represents the mean of triplicate determinations, and vertical bars indicate the mean ± S.E. (D) Interaction of rER with estrogen response elements (EREs). The rER and tER were expressed in CHO-K1 cells as a fusion protein with the herpes simplex virus VP16 activation domain, along with 4xERE-driven luciferase reporter. Three types construct were used for assay: ER-D containing DBD, hinge and LBD; ER-H containing hinge and LBD, ER-Dmt containing DBD, hinge and AF-2 mutated LBD. Fold activation indicates luciferase activity relative to the vector-only control.

cells with a pG5-luc reporter construct, using charcoal-stripped serum to eliminate the potential for spurious ligand activation. As expected, there was no activation above background by the ERα from the freshwater turtle (*Pseudemys nelsoni*) in the absence of ligands. Increasing the concentration of E2 (1 pM-100 nM) revealed a concentration-dependent reporter activation. In contrast, the rock shell ER-like protein was constitutively active. It activated transcription 60 -fold above background when no ligand was added. Addition of E2 had no further effect on reporter activity (Figure 3A). We also treated cells with various steroids (each at 10 nM). The turtle ERα showed the expected activation by estrogens, E2, and ethinyles-

tradiol (EE2). In contrast, reporter activity in response to the rock shell ER-like protein was not changed by the estrogens (E2 and EE2), progesterone, androgens (testosterone and trenbolone), or corticosteroid (corticosterone) (Figure 3B). The rock shell ER-like protein's constitutive activity is not an artifact of saturating assay conditions because increasing the quantity of the rock shell ER-like protein expression plasmid led to an increase in levels of reporter expression when all other assay conditions were held constant (data not shown). Furthermore, we found that the full-length rock shell ER-like protein also constitutively activat-ed an ERE-driven luciferase reporter and did not respond to E2 treatment (data not shown).

Next, we examined the necessity of activation function-2 (AF-2) domain for the transactivation of rock shell ER-like protein (Figure 3, C, D). ER contains two distinct activation regions, AF-1 and AF-241-43. AF-1 is located in the N-terminal A/B domain and exerts ligand-independent transcriptional activity in re-sponse to phosphorylation[44-46]. AF-2 is located in the C-terminal ligand-binding domain and mediates ligand-dependent transcriptional activity[47]. AF-1 and AF-2 activate transcription independently or synergistically and act in a promoter-specific and cell-specific manner[48]. To date, two classes of nuclear receptor coactivator complexes have been identified that directly interact with AF-2 in a ligand-dependent manner. The first contains CBP/p300, the p160 nuclear receptor coactivator family (SRC-1/TIF2/AIB1), an RNA coactivator (SRA), and probably other unknown components[49,50]. This complex facilitates decondensation of the chromatin via the histone acetyltransferase activity of several of its components[51,52]. The second coactivator complex includes proteins of the SMCC/TRAP/DRIP/ARC/Mediator class, and allows the physical link between ER and the general transcription apparatus, facilitating the activation of polymerase II[53]. Cell-type specific activity of both AFs was suggested by results from a specific expression of distinct coactivators[54]. However, the majority of the coactivators are widely expressed at similar levels in most cells[53,54]. Several of the coactivators primarily identified as AF-2 specific have now been shown to also interact with the N-terminal region of ER and to mediate AF-1 activity[48,55]. Therefore, despite considerable advances in understanding the mechanisms allowing the receptor to modulate the transcription of a target gene, no clear scheme is emerging with regard to the differential sensitivity of cell types to AF-1 and AF-2.

Overall, it is difficult to draw firm conclusions regarding the mechanism of organotin action in gastropods, due to deficiencies in our understanding of their endocrinology and developmental biology. An important first step will be the elucidation of the key steroid hormones involved in sex determination, information regarding the enzymology of steroid hormone synthesis and identification of the steroid hormone receptors and their mode of action.

Adipogenesis Stimulation by Organotins in Vertebrates

Recent work has shown that aromatase mRNA levels can be down-regulated in human ovarian granulosa cells by treatment with organotins or ligands for the nuclear hormone receptors, RXR or peroxisome proliferator activated receptor gamma (PPARγ)[56-58]. Furthermore, as mentioned above, the gastropod *T. clavigara* RXR homolog is responsive to 9-*cis* RA and TBT, and 9-*cis* RA can also induce imposex, suggesting a conserved transcriptional mechanism for TBT action across phyla[35]. RXR and PPARγ are ligand-modulated transcription factors that belong to the nuclear hormone receptor superfamily. This is a group of ~150 members (there are 48 human genes) that includes the ER, AR, GR, thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic acid receptors (RARs and RXRs), PPARs and numerous orphan receptors. We found the similar effects of TBT and RXR/PPARγ ligands on mammalian aromatase mRNA expression intriguing. This led us to hypothesize that TBT could be acting as a nuclear receptor ligand to exert some of its biological effects as a transcriptional regulation of gene expression.

Our results demonstrated that TBT induces adipogenesis, *in vitro* and *in vivo*, through its ability to act as a novel, high-affinity ligand for RXRα and PPARγ[59]. Analysis of structure-activity profiles revealed distinct structural preferences for organotins in their ability to activate both receptors. This analysis also showed that organotins of relatively diverse 3-D structures (e.g., TBT and TPT) efficaciously activate these receptors. The ability of the same compounds to bind to, and activate both RXR and PPARγ is surprising, and not easy to reconcile with the classical models of ligand binding for these receptors. It is possible that organotins will interact somewhat differently than previously described RXR/PPARγ ligands. However, the strong binding affinity of organotins for the receptors, coupled with the ability of organotins to displace high-affinity RXR and PPAR ligands suggest that organotins are potent and efficacious ligands for both RXRs and PPARγ. We also note that TBT activates both receptors at nanomolar concentrations, whereas other mechanisms of toxicity, e.g., direct inhibition of aromatase activity, typically occur at micromolar levels. This also supports the model

that organotins may be more likely to affect transcription than other cellular processes at environmentally-relevant concentrations.

Another interesting point is the ability of TBT to act as a dual ligand for permissive heterodimers such as RXRα : PPARγ. These heterodimers can be activated by specific ligands for either receptor individually. Additive or synergistic effects have been observed for permissive heterodimers when ligand for both partners is added together. This raises the possibility that TBT might itself elicit additive or synergistic effects on receptor activation, perhaps even in conjunction with natural ligands. The ability of organotins to activate permissive RXR heterodimeric partners suggests that organotins may have even wider effects on nuclear receptor signaling and endocrine disruption, e.g. LXR and NURR1, suggests that organotins may act more widely to disrupt multiple nuclear receptor mediated hormonal signaling pathways.

The effects of organotin activation of the RXR : PPARγ signaling pathway are predictable and reflect known aspects of RXR/PPARγ biology. The RXR : PPARγ heterodimer plays a central role in regulating adipocyte differentiation and lipid storage and is a key regulator of whole body lipid metabolism. Activation of PPARγ promotes the expression of genes that increase fatty acid storage and inhibits expression of genes that induce lipolysis in white adipose tissue[60]. PPARγ ligands such as the anti-diabetic thiazolidinediones increase insulin sensitivity through these effects on the adipocyte, sensitizing muscle and liver to insulin and thereby reversing insulin resistance in the whole body[61]. An important and undesirable consequence of this increase in whole body insulin sensitivity is that fat mass is increased through the promotion of triglyceride storage in adipocytes coupled with depot-specific remodeling and increase in adipocyte numbers increase following thiazolidinedione treatment[62-64]. Therefore, PPARγ agonists comprise a class of pharmaceutical therapies for type 2 diabetes that can also promote obesity by increasing fat storage. The ability of thiazolidinediones to increase adipocyte number and fat mass suggests that TBT exposure, which activates the same receptors, could affect obesity at any time in life. It is currently an open question whether the increased adiposity resulting from organotin exposure is due to an increase in adipocyte precursor cell number, enhanced adipocyte differentiation from the same number of precursors, an increase in adipocyte size without an increase in number or some combination of these.

The conventional wisdom suggests that high calorie modern diets, coupled with reduced physical activity are the major, or only cause of the dramatic rise in obesity rates worldwide[65]. Although the role played by genetic components is not completely clear, there is little doubt that genetic variation affects individual weight gain. However, it is difficult to imagine a scenario where genetic variation could underlie the rapid worldwide increase in obesity. It is more reasonable to suggest that interaction with the modern environment exposes underlying genetic differences that affect obesity. The Barker hypothesis postulates that *in utero* fetal nutritional status is a potential risk factor for metabolic syndrome diseases[66-70]. Developmental programming of a thrifty phenotype limits the range of subsequent responses to environmental factors such as diet and exercise[71]. Experiments in animal models support this hypothesis[72]. Plausible mechanisms include imprinting of obesity sensitive hormonal pathways or changes in cell type and number e.g. adipocytes, established during development.

An alternative model suggests that the environment plays another role in obesity. The increase in obesity rates parallels the rapid growth in the use of industrial chemicals over the past 40 years. Therefore, it is plausible to associate exposure to environmental chemicals in utero, or throughout ones lifetime with the obesity epidemic. We developed an “obesogen” model that predicts the existence of xenobiotic chemicals that inappropriately regulate lipid metabolism and adipogenesis to promote obesity. Several recent studies provide “proof-of-principle” for the obesogen hypothesis. In addition to organotin exposure, treatment with environmental estrogens such as bisphenol A and nonylphenol can promote adipocyte differentiation or proliferation in murine cell lines[73,74]. Furthermore, epidemiological studies link maternal smoking during pregnancy to an elevated risk of childhood obesity[75-79].

Discussion

Organotins such as TBT and TPT act as RXR activators, resulting in the development of imposex in the rock shell. They also act as chemical stressors or “obesogens” that activate RXR : PPARγ signaling to promote long term changes in adipocyte number and/or lipid homeostasis following developmental or chronic lifetime exposure in vertebrates.

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