Biochemical Pharmacology 81 (2011) 819–824

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Tributyltin chloride induces ABCA1 expression and apolipoprotein A-I-mediated cellular cholesterol efflux by activating LXRalpha/RXR

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ARTICLE INFO

Article history:
Received 9 November 2010
Accepted 30 December 2010
Available online 8 January 2011

Keywords:
Tributyltin chloride
Retinoid X receptor
Liver X receptor
ABCA1
HDL

ABSTRACT

Organotins, including tri-butyltin chloride (TBTC), are widely used in agricultural and chemical industries and cause persistent and widespread pollution. TBTC has been shown to activate nuclear receptor retinoid X receptor (RXR)/PPARγ signaling by interacting with RXR to modulate adipogenesis. However, whether TBTC affects liver X receptor (LXR)/RXR activity and subsequently the expression of cholesterol mobilizing genes is not known. In this study, we evaluated the ability of TBTC to activate LXR/RXR and ABC transporter A1 (ABCA1) expression. ABCA1 plays a critical role in HDL generation, maintaining cholesterol homeostasis, and cholesterol accumulation-induced diseases, such as atherosclerosis and pancreatic islet dysfunction. In a reporter gene assay, TBTC activated LXRα/RXR but not LXRβ/RXR. In mouse macrophage RAW264 cells, TBTC activated the ABCA1 promoter in an LXR-responsive element dependent manner and increased ABCA1 mRNA expression. TBTC augmented ABCA1 protein levels and apolipoprotein A-I-dependent cellular cholesterol efflux (HDL generation). The LXR-target fatty acid synthase and Spα mRNA levels were also increased by TBTC exposure. We conclude that TBTC has the ability to activate permissive LXRα/RXR signaling and thereby modulate cellular cholesterol efflux.

1. Introduction

Organotin compounds have been widely used as plastic stabilizers, and antifouling and antifungal agents in industry and agriculture. As they cause persistent and widespread environmental pollution, their bioaccumulation in higher organisms is now a problem of great concern. The organotin tri-butyltin chloride (TBTC) and its derivatives are known as environmental endocrine disruptors [1,2]; TBTC exposure causes imposex- the abnormal induction of male characteristics- in many marine species [3,4]. In mammals, organotin exposure has been shown to induce adipogenesis both *in vitro* and *in vivo* [5,6], and to reduce fertility [7,8]. Decreased aromatase activity resulting in increased testosterone levels is thought to be associated with the disruption of the endocrine system [4,9].

Recent studies showed that TBTC is a potential dual agonist of RXR and PPARγ [6] and exerts its adipogenic effects by activating these receptors, which in turn alters endocrine function [5,6,10]. A structural analysis study demonstrated that TBTC activates RXR/PPARγ heterodimers at nanomolar concentrations, primarily by interacting with RXR [11]. It is therefore likely that TBTC activates the liver X receptor (LXR)/RXR heterodimer, which is known as the permissive RXR heterodimer that is activated by agonists of either RXR or its partner receptor, or both in a more-than-additive fashion [12,13]. However, we and others found that whether LXR/RXR is permissive or non-permissive is ambiguous, and that the ability of RXR agonists to activate LXR/RXR varies depending on their chemical structures [14,15]. Indeed, we showed that the synthetic RXR agonist HX630 is incapable of activating LXR/RXR but is able to activate PPARγ/RXR, while the RXR agonist PA024 can activate both heterodimers [15].

LXRα and LXRβ serve as cholesterol sensors and regulate genes for efflux, transport, and catabolism of cholesterol, as well as triglyceride production [16]. RXR modulators are implicated in metabolic diseases [17,18], but whether TBTC affects cholesterol homeostasis by modulating LXR/RXR signaling is unknown. In this study, we evaluated the ability of TBTC to activate LXR/RXR and modulate LXR-target gene expression. The expression of the ABC transporter A1 (ABCA1) gene has been shown to be stimulated by direct binding of LXR/RXR to its promoter region [19]. ABCA1 plays

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doi:10.1016/j.bcp.2010.12.023

a dominant role in maintaining cholesterol homeostasis by mediating HDL assembly through its transport of cellular cholesterol and phospholipids to extracellular apolipoprotein A-I (apo A-I) [20]. ABCA1 levels are critical determinants of plasma HDL levels [20–22] and also affect many pathological processes [23–25]. ABCA1 deficiency promotes cholesterol accumulation in tissues, accelerates atherosclerosis, and is associated with islet dysfunction and loss-of insulin secretion [23,24]. In addition, ABCA1 expression in Sertoli cells influences male fertility [25]. In this study we show that TBTC augments ABCA1 expression and its function by activating LXR/RXR.

2. Materials and methods

2.1. Chemical compounds

Tri-butyltin chloride (TBTC) (Wako Pure Chemicals, Tokyo, Japan) and PA024 [26] stock solutions were prepared using dimethyl sulfoxide (DMSO) (Merck, Germany) and were then diluted in the medium to a final concentration as indicated.

2.2. Cell culture and real time quantitative RT-PCR

The African green monkey kidney SV40 transformed cell line COS1 and mouse macrophage-like cell line RAW264 were obtained from the JCTB Cell Bank (Osaka, Japan) and the Riken Gene Bank (Tsukuba, Japan), respectively, and maintained in 10% fetal calf serum-supplemented DMEM and DMEM/F-12 (1:1), respectively. To evaluate the effect of TBTC on mRNA expression, RAW264 cells were treated in medium containing 0.1% bovine serum albumin (BSA) in the presence or absence of TBTC for 20 h. The RNA samples were treated with DNase according to the manufacturer's protocol (Qiagen). Relative expression levels of mRNA were determined using a TaqMan one-step RT-PCR MasterMix Reagent Kit and an ABI Prism7300 sequence detection system (Applied Biosystems). Primer/probe sequences used were as follows: FAS forward primer, 5′- CTCACGCTGCGGAAACTTCAG-GAAATG-3′; reverse primer, 5′- GAGACGTGTCACTCCTGGACTTG-3′; probe, 5′- FAM- CTCACGCTGCGGAAACTTCAGGAAATG-TAM RA-3′;SPα forward primer, 5′- CAGTGTGAGAACCCAGACAG TGA-3′; reverse primer, 5′- GCCCCGGGCCATCTACT-3′; probe, 5′- FAM-CTCCTCTTCATTCCAGAGGATGTGCGTC-BHQ. The primer/probe sequences for mouse ABCA1 were described previously [27]. Expression data were normalized to18S rRNA levels, and presented as the fold difference between treated and untreated cells.

2.3. Plasmid constructs

Plasmids for an LXRE-driven luciferase reporter were constructed using cDNAs containing four copies of LXRE from the sterol response element binding protein-1c promoter. cDNAs encoding full-length human RXRα, LXRα, LXRβ were PCR-cloned and inserted into the mammalian expression vector pcDNA3.1 (Invitrogen). A mouse peripheral-type ABCA1 promoter (−1238/+57 of exon 1)-luciferase vector (pABCA1-Luc) has been described previously [28]. An ABCA1 promoter construct carrying mutations in the LXR-responsive element (LXRE) (pABCA1:mut-LXRE-Luc) was prepared as described previously [28].

2.4. Transient transfections, reporter gene assays, and in vitro coactivator association assays

For the ABCA1 promoter assay, RAW264 cells were transfected with 1.0 μg of pABCA1-Luc or pABCA1:mut-LXRE-Luc and 67 ng Renilla luciferase vector (phRL-SV40) (Promega) with SuperFect (Qiagen) in 12-well plates. An empty pcDNA3.1 expression vector was used to maintain equal amounts of DNA for each transfection. Firefly luciferase activity was normalized to Renilla luciferase for each well. Activation studies on LXR and PPAR were performed as described previously [29]. COS1 cells were transfected with 250 ng pLXRE-tk-Luc, 6.25 ng each of pcDNA3.1-LXR and pcDNA3.1-RXRα, and 125 ng SV-β-galactosidase control vector (Promega). Three hours after transfection, cells were exposed to TBTC in medium containing 0.1% BSA for 24 h. 20 μM Compactin and 10 μM mevalonic acid were added to the medium to deplete endogenous LXR agonist. Luciferase and β-galactosidase activities were determined in cell lysates. Ligand-induced association of LXRα with a coactivator SRC-1 peptide *in vitro* was measured using RCAS assay system (Enbiotec Laboratories, Tokyo) according to the manufacture's instruction.

2.5. Measurement of cellular cholesterol efflux to apoA-I

RAW264 cells were sub-cultured in 6-well plates and treated with 300 μmol/l cAMP and indicated reagent treatment in DMEM/F-12 (1:1) containing 0.1% bovine serum albumin for 16 h, and then chased with the same medium in the presence or absence of 15 μg/ml human apoA-I for an additional 24 h. Cholesterol was extracted from the medium and the cells with chloroform/methanol (2:1, v/v) and hexane/isopropanol (3:2, v/v), respectively, and cholesterol amounts were determined using a specific enzymatic method [30].

2.6. Western blot analysis

ABCA1 protein expression was examined by immunoblotting. Cultured cells were first washed twice with ice cold PBS and 200 μl dissolution buffer (10 mM Tris–HCl (pH8.5), 0.9 M urea, 0.2% TX-100, 25 μg/ml ALLNand 2% SDS) with protease inhibitors (Calbiochem Protease Inhibitor Cocktail Set III;1:100) was then added to each well on ice. After centrifugation at 10,000 × *g* for 5 min, proteins in the supernatant were separated by electrophoresis on 5–20% SDS-PAGE, and then electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratory, Hercules, CA). After blocking with 4% BlockAce, the membrane was incubated for 1 h at room temperature with anti-ABCA1 (Novus Biologicals, Littleton, CO) and then with an anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences) for 1 h. ABCA1 was visualized by using a SuperSignal West Femto Substrate kit (Thermo Scientific, Rockford, IL).

2.7. Statistical analysis

Data were analyzed by ANOVA followed by the Student–Newman–Keuls method. Statistical significance was established at the $P < 0.05$ level.

3. Results

3.1. TBTC can activate LXRα/RXR

The ability of TBTC to activate LXRα/RXR was tested in COS-1 cells transfected with a LXRE-driven luciferase-reporter vector (Fig. 1A). When LXRα/RXR plasmids were co-transfected into cells, TBTC at up to 100 nM markedly increased luciferase activity (Fig. 1B). Expression of LXRβ/RXR augmented the luciferase transcription in the vehicle-treated control, but the response induced by TBTC was small and equivalent to that caused by endogenous receptors. TBTC at concentrations higher than 1 μM was toxic and caused decreased expression of control Renilla

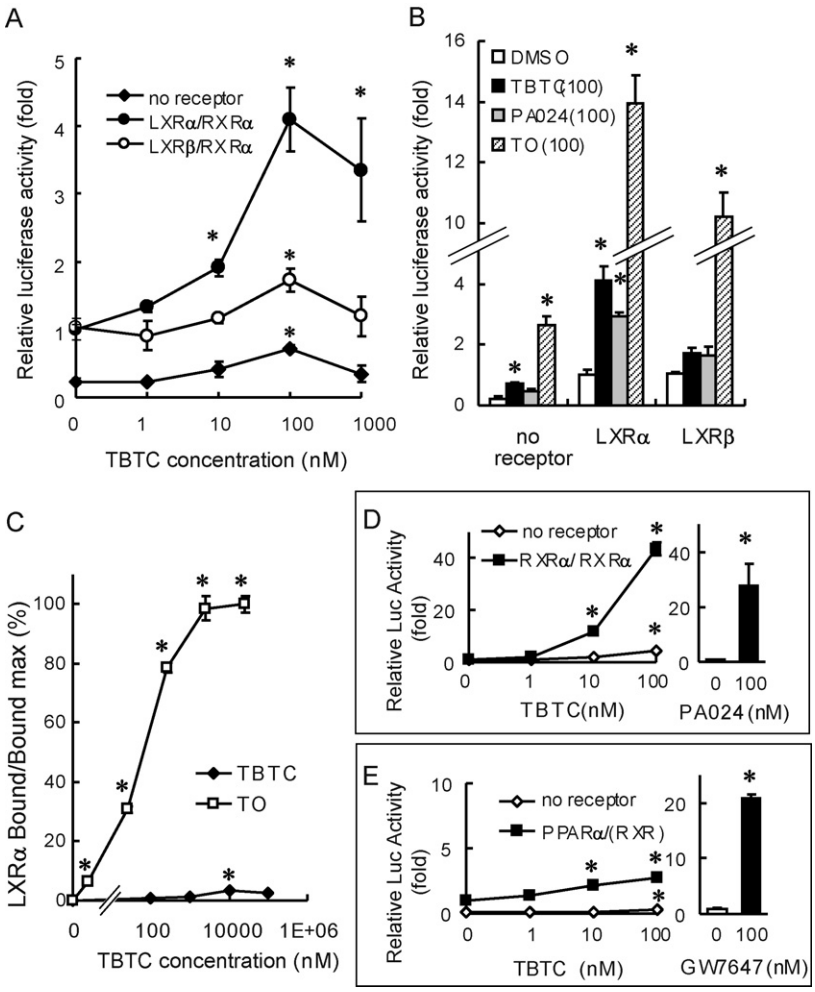

Fig. 1. TBTC augments LXR/RXR transactivation. COS-1 cells were transfected with LXREx4-tk-Luc reporter plasmid together with a β-gal internal control in the absence or presence of expression plasmids for human LXRα and RXRα, or LXRβ and RXRα. The cells were treated with the indicated concentrations of TBTC (A), 100 nM TBTC, PA024, or TO (TO901317) (B) in the presence of 20 μM compactin and 10 μM mevalonic acid. Luciferase activity in the cell extract was normalized using β-galactosidase and expressed as fold induction relative to vehicle-treated cells. (C) Ligand-induced association of LXRα with a SRC-1 peptide was determined using RCAS assay system. (D and E) TBTC stimulates RXR/RXR (D) and PPARα/RXR (E) transactivation. Cells were transfected with PPRE-tk-Luc reporter plasmid together with a β-gal internal control in the absence or presence of either human PPARα or RXRα plasmid alone, and treated with agents at indicated concentrations. The data represent the average ± S.D. of three experiments. Significantly different from respective control or vehicle-treated cells (*).

luciferase vector. TBTC at 100 nM elicited LXRα/RXR activation comparable to that induced by the same concentration of RXR agonist PA024 [15]. We confirmed that the LXR agonist TO901317 (100 nM) efficiently activated LXRβ/RXR as well as LXRα/RXR. In a coactivator recruitment assay *in vitro*, TO901317 greatly induced association of LXRα with a SRC-1 peptide, whereas TBTC had negligible effect (Fig. 1C). Furthermore, when RXRα plasmid alone was transfected into COS-1 cells along with a DR-1-element (PPRE)-driven luciferase reporter vector, TBTC markedly increased luciferase transcription (Fig. 1D), indicating activation of RXRα homodimers. These findings indicate that TBTC is able to activate LXRα/RXR by interacting with RXR.

The ability of TBTC to activate PPARα/RXR was tested. When a PPARα plasmid alone was transfected along with a PPRE-driven luciferase vector, a PPARα agonist GW7647 greatly increased luciferase activity (Fig. 1E), suggesting that PPARα/RXR heterodimer made from exogenous PPARα and endogenous RXR was able to enhance the reporter gene transcription. To avoid forming exogenous RXRα-derived homodimer which could stimulate PPRE, we employed this reporter assay system. TBTC slightly enhanced transcription (by ca. 2.7-fold), indicating that TBTC has the ability to activate PPARα/RXR heterodimer.

3.2. TBTC enhances LXR -target gene ABCA1, SPα, and FAS expression

We examined the effect of TBTC on the mRNA levels of LXR targets ABCA1 and FAS, and the LXRα-specific target anti-apoptotic factor SPα (AIM/Api6) [31] in the mouse macrophage cell line RAW264. Treatment of RAW264 cells with TBTC or PA024 up to 100 nM resulted in a marked increase in ABCA1 mRNA expression (Fig. 2). TBTC also increased FAS and SPα mRNA expression.

3.3. TBTC activates ABCA1 transcription in an LXRE-dependent manner

To determine whether TBTC enhances ABCA1 gene transcription, we examined the effect of TBTC on ABCA1 promoter activity by transfecting RAW264 cells with an ABCA1 promoter (−1238/+57 of exon1)-luciferase construct [28]. As shown in Fig. 3A, treatment of cells with both TBTC and PA024 markedly increased ABCA1 promoter-dependent transcription. However, the increase caused by TBTC was lost when a mutation was introduced into the LXR-responsive element (LXRE) in the promoter (Fig. 3B). Neither LXRα mRNA nor protein level in RAW264 cells was affected by 100 nM TBTC (data not shown). These findings indicate that TBTC activates

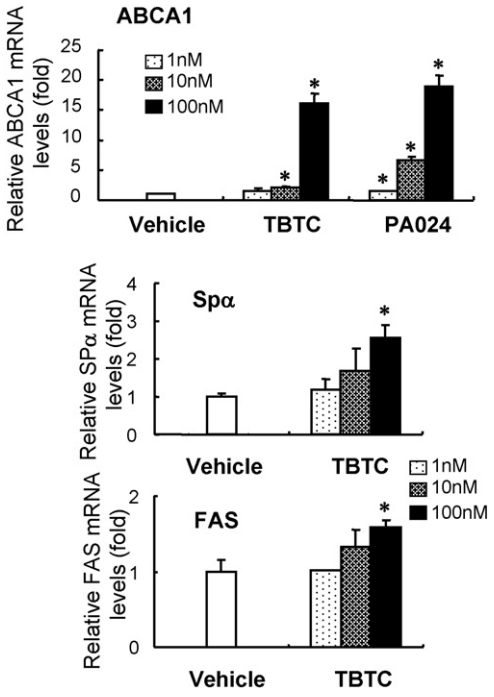

Fig. 2. TBTC increases mRNA expression of LXRα target genes ABCA1, SPα and FAS. RAW264 cells were treated for 20 h with the indicated concentrations of TBTC or PA024. ABCA1, SPα, and FAS mRNA levels were measured by quantitative real-time RT-PCR analysis, standardized against 18S rRNA levels, and expressed as fold induction relative to vehicle-treated cells. The values represent the average ± S.D. from three experiments. Significantly different from vehicle-treated cells (*).

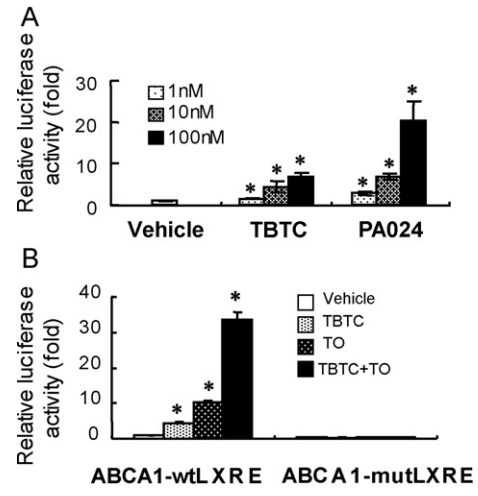

Fig. 3. TBTC activates the ABCA1 promoter in an LXRE-dependent manner. (A) RAW264 cells were transfected with a pABCA1-Luc reporter plasmid together with a phRL-SV40 internal control and treated with the indicated concentrations of TBTC and PA024. (B) RAW264 cells were transfected with pABCA1-wtLXRE-Luc (ABCA1-wtLXRE) or pABCA1-mutLXRE-Luc (ABCA1-mutLXRE) reporter plasmid together with a phRL-SV40 internal control, and treated with vehicle, 100 nM TBTC, 100 nM TO (TO901317), or both compounds. Luciferase activity in the cell extract was normalized using Renilla luciferase activity and expressed as fold induction relative to vehicle-treated cells. The data represent the average ± S.D. of three experiments. Significantly different from vehicle-treated cells (*).

ABCA1 promoter in an LXRE-dependent manner by directly stimulating LXRα/RXR transactivation.

3.4. TBTC increases ABCA1 protein levels and apoA-I-mediated cholesterol efflux

ABCA1 protein expression in RAW264 cells is too low to evaluate its function at a basal level, but ABCA1 levels can be dramatically increased in the presence of cAMP [27]. We therefore examined TBTC-dependent changes in cellular ABCA1 mRNA and protein levels in cAMP-treated RAW264 cells. Treatment of cells with cAMP led to ca. 60-fold induction of ABCA1 mRNA level (*data not shown*). As shown in Fig. 4A, ABCA1 mRNA expression was up-regulated by treatment with TBTC or the LXR agonist TO901317, although their effects were attenuated in cAMP-treated cells. TBTC combined with TO901317 caused a more than additive effect. Immunoblotting analysis of cell lysates demonstrated that TBTC, and TBTC together with TO901317, increased ABCA1 protein levels (Fig. 4B).

Since ABCA1 generates HDL particles by mediating cellular cholesterol efflux into extracellular apoA-I [20], we examined the effect of TBTC on apoA-I-dependent cellular cholesterol efflux in cAMP-treated RAW264 cells. As shown in Fig. 4C, TBTC signifi-

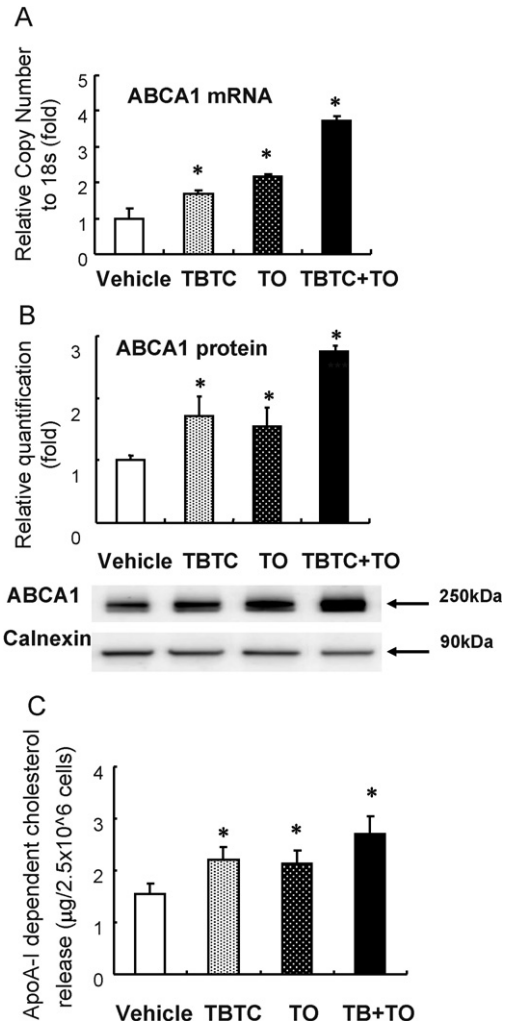

Fig. 4. TBTC enhances ABCA1 mRNA and protein expression and apoA-I mediated cholesterol efflux in cAMP-treated RAW264 cells. RAW264 cells were treated with vehicle, 100 nM TBTC, 100 nM TO (TO901317), or both compounds in the presence of 300 μM diBu-cAMP for 16 h. (A) ABCA1 mRNA expression levels were measured by quantitative real-time RT-PCR analysis, standardized against 18S rRNA levels, and expressed as fold induction relative to vehicle-treated cells. The data represent the average ± S.D. (n = 3) of a typical series of 3 experiments. (B) Western blot analysis of ABCA1 in cells treated with indicated reagents for 24 h. Membrane fractions from RAW264 were prepared, and aliquots (20 μg protein per lane) were subjected to immunoblot analysis for ABCA1 and the loading control calnexin. (C) RAW264 cells treated with reagents for 16 h were then incubated in the same medium in the presence or absence of 15 μg/ml human apoA-I for an additional 24 h. ApoA-I dependent release of cholesterol into the medium was analyzed. The data represent the average ± S.D. (n = 3) of a typical series of 3 experiments. Significantly different from vehicle-treated cells (*).

cantly increased apoA-I-dependent release of cholesterol, with addition of TO-901317 further potentiating this effect.

4. Discussion

In this study, we evaluated the ability of TBTC, a known RXR agonist, to activate LXR/RXR and modulate LXR-target gene expression. Although LXR/RXR is known as a permissive heterodimer [12,13], we and others have found that certain synthetic RXR agonists act as non-permissive ligands for LXR/RXR [14,15]. In the present study, we showed that TBTC at up to 100 nM activated LXRα/RXR potently but not LXRβ/RXR (Fig. 1), indicating that TBTC acts as a permissive ligand for LXRα/RXR but is a non-permissive ligand for LXRβ/RXR.

Furthermore, TBTC had the ability to increase LXRs-target genes ABCA1 and FAS, and LXRα-target SPα mRNA expression in RAW264 cells (Fig. 2). TBTC activated the ABCA1 promoter in an LXRE-dependent manner (Fig. 3) and augmented ABCA1 mRNA expression. We previously showed that the ability of RXR agonists to activate LXR/RXR in RAW264 cells can be evaluated without the influence of PPARγ/RXR-mediated LXRα induction, because PPARγ levels are negligible in this cell line [15]. Indeed, there were no detectable changes in LXRα mRNA and protein levels by TBTC treatment. Collectively, these findings indicate that TBTC augments ABCA1 transcription by directly stimulating LXRα/RXR. A crystallographic study showed that TBTC binds to RXR in PPARγ/RXR heterodimer [11]. We showed that TBTC is able to activate RXRα/RXRα homodimer (Fig. 1D). Furthermore, TBTC lacked the ability to induce coactivator association with LXRα (Fig. 1E). Thus, TBTC activates LXRα/RXR by interacting with RXR.

We further showed that TBTC increased ABCA1 protein levels (Fig. 4A and B), leading to enhanced HDL generation (Fig. 4C). Of note, the TBTC concentrations required for ABCA1 induction are comparable to those of the potent synthetic RXR agonist, PA024 [15] (Figs. 2 and 3). Taken together, our findings indicate that TBTC at nanomolar concentrations has the ability to stimulate cholesterol efflux and thereby modulate cholesterol homeostasis by activating LXRα/RXR.

LXRα and LXRβ share a high degree of amino acid similarity and are similarly activated by physiological oxysterol ligands [16]. Although their tissue distributions differ, both receptors play crucial roles in the transcriptional control of lipid metabolism [16]. In particular, ABCA1 plays essential roles in HDL biogenesis and the reverse cholesterol transport pathway [20]. Absence of functional ABCA1 in Tangier disease and in knockout mice results in a near absence of plasma HDL, whereas enhanced expression of an ABCA1 transgene in mice causes increased plasma HDL concentration [21,22]. HDL is thought to play a main role in exporting cholesterol to maintain its homeostasis both at a cellular and systemic level [22]. ABCA1-mediated HDL generation may reduce excess intracellular cholesterol, which would contribute to prevention or regression of the initial stages of atherosclerosis [21–23]. In addition, the functional disruption of ABCA1 is closely associated with insulin secretion [24] and male fertility [25]. Our findings that TBTC induces ABCA1 expression and cholesterol efflux may suggest that TBTC has the ability to affect cholesterol homeostasis via modulation of ABCA1 expression. Furthermore, LXRs are also involved in regulating inflammatory responses that are associated with the development of atherosclerosis [32]. In addition, LXR activation stimulates lipogenesis by upregulating SREBP-1c expression [16]. Collectively, these findings suggest that TBTC could be implicated in metabolic diseases.

The ability of RXR agonists to activate LXR/RXR has been shown to vary according to chemical structure and cell type [14,15]. Agonist binding to nuclear receptors produces conformational changes that allow dissociation of co-repressors and association of co-activators with the receptors [33]. When a permissive agonist binds to the RXR subunit of heterodimers, this agonist must cause similar conformational changes in the partner receptor that does not have ligand bound. RXR agonists are therefore called "phantom ligands" [34]. Amino acid residues that transduce the allosteric signal across the heterodimer interface have been identified, and the RXR agonist chemical structure and the type of partner receptor are thought to contribute this allosteric network and thereby determine permissivity [13]. TBTC was shown to induce association between RXRα and coactivator TIF2 *in vitro* [6]. It is also proposed that cellular expression level of coactivators may be associated with cell-type or tissue-specific effects of RXR agonists [35]. We found that TBTC was permissive for LXRα/RXR and PPARα/RXR as well as for PPARγ/RXR [6], but non-permissive for LXRβ/RXR. These findings suggest the possibility that TBTC is an ambiguous RXR agonist that may exert variable effects depending on the partner receptors and cell type examined.

Acknowledgments

This work was supported in part by a grant from the Japan Health Sciences Foundation, and Grant-in Aid for Scientific Research 20590116 from Japan Society for the Promotion of Science.

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