Chemosphere 87 (2012) 782–788

Contents lists available at SciVerse ScienceDirect

Chemosphere

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

Tetrabromobisphenol-A disrupts thyroid hormone receptor alpha function *in vitro*: Use of fluorescence polarization to assay corepressor and coactivator peptide binding

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ARTICLE INFO

Article history:
Received 2 September 2011
Received in revised form 16 December 2011
Accepted 30 December 2011
Available online 25 January 2012

Keywords:
Thyroid disrupting chemicals
Thyroid hormone receptor
Transcriptional coregulators
Environmental chemicals
Tetrabromobisphenol-A
Fluorescence polarization

ABSTRACT

Thyroid hormone receptors (TRs) recruit corepressor or coactivator factors to the promoters of target genes to regulate their transcription. Corepressors such as nuclear hormone receptor corepressor (NCoR) are recruited by unliganded TRs, whereas coactivators such as steroid receptor coactivator-2 (SRC2) are recruited when triiodothyronine (T3) is bound to TRs. These coregulator proteins interact with the ligand binding domain (LBD) of TRs via short, conserved peptide sequences that can be used to probe the conformational changes induced in TR LBD by TR ligands. Recombinant LBD of the human TRα1 isoform (hTRα1 LBD) was produced as a fusion with glutathione S-transferase, and used to develop assays based on fluorescence polarization to quantify the binding of either NCoR- or SRC2-derived fluorescent peptides to the hTRα1 LBD. The optimum concentrations of recombinant hTRα1 LBD, and of peptide probes were adjusted in order to produce the greatest possible T3-dependent signal variations in fluorescence polarization. Under these conditions, T3 induced a dose-dependent decrease in NCoR peptide binding, and a reciprocal dose-dependent increase in SRC2 peptide binding, in both cases at similar 50%-effective doses. The TR agonists triiodothyroacetic acid and thyroxine were also effective in preventing NCoR peptide binding and increasing SRC2 peptide binding, whereas reverse-triiodothyronine was less efficient and the biologically inactive thyronine had no effect on either process. These experiments validate cell-free assays based on the use of binding of corepressor or coactivator peptide probes, as measured by fluorescence polarization, for investigating the conformational changes of TRα1 LBD induced by potentially TR-interfering compounds. Both these methods were used to elucidate the mechanism of the disrupting effects of tetrabromobisphenol-A (TBBPA) on the hTRα1 LBD conformation related to the transcriptional activity of the receptor. TBBPA is a flame retardant that is released into the environment, and is a suspected disrupter of thyroid homeostasis. The present results indicate that TBBPA did indeed interfere with the ability of the hTRα1 LBD to bind both NCoR and SRC2. TBBPA behaved similarly to T3 in promoting the release of NCoR from LBD, whereas it failed to promote LBD interactions with SRC2. However, it did reduce the T3-induced interactions between LBD and the coactivator peptide. This study therefore suggests that TBBPA in the micromolar range can affect the regulation of transcription by both the apo- and the holo-TRα1, with potential disruption of the expression of genes that are either up- or down-regulated by T3.

1. Introduction

Chemicals that interfere with the various activities of natural hormones are known as endocrine disrupting chemicals (EDCs). These frequently-detected environmental pollutants can interfere with various aspects of the endocrine system, ranging from hormone synthesis to hormone receptor activities (Diamanti-Kandarakis et al., 2009). Initially, investigation focused on the effects of EDCs on steroid hormone nuclear receptors, particularly estrogen receptors. However, it is now known that several classes of EDCs can also disrupt the action of thyroid hormones (THs). Alterations of circulating TH levels are well-documented consequences of

Abbreviations: EDC, endocrine disrupting chemical; FITC, fluorescein isothiocyanate; FP, fluorescence polarization; GST, glutathione S-transferase; hTRα1, human thyroid hormone receptor, α1-isoform; LBD, ligand-binding domain; NCoR, nuclear receptor corepressor; NCoRp, NCoR peptide; rT3, 3,3′,5′-triiodo-L-thyronine; SEM, standard error of the mean; SMRT, silencing mediator of the retinoid and thyroid hormone receptor; SRC, steroid receptor coactivator; SRC2p, SRC2 peptide; T3, 3,3′,5-triiodo-L-thyronine; T4, L-thyroxine; TBBPA, 3,3′,5,5′-tetrabromobisphenol A; TDC, thyroid disrupting chemicals; TH, thyroid hormone; TRIAC, 3,3′,5-triiodothyroacetic acid.

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doi:10.1016/j.chemosphere.2011.12.080

exposure to thyroid disrupting chemicals (TDCs). Consequently, attention has focused on the effects of TDCs on thyroid histology, TH synthesis, TH blood transport, and TH catabolism. It is also recognized that various chemicals can affect TRs and their transcriptional activity by acting as thyroid hormone receptor (TR) agonists or antagonists (Zoeller, 2005; Jugan et al., 2010). Evidence for interference of various EDCs with TR-dependent transactivation was recently reported by Hofmann et al. (2009), using a cell-based, TH-responsive, luciferase-based reporter assay.

The TRs are nuclear receptors. They are encoded by two genes that generate TRα1 and TRβ1/2, the major TH-binding isoforms in humans (Yen, 2001). They bind triiodothyronine (T3), the active intracellular form of TH, with similar affinities, but their expression levels vary in different tissues. Numerous developmental processes and key metabolic pathways are regulated by TRs and T3 by activating or repressing target gene transcription. TRα1 and TRβ1/2 display both overlapping and distinct functions. Thus, TRα1 is crucial for the postnatal maturation of brain, bone, and intestine, and for cardiac function, whereas TRβs mainly control inner ear and retina development, liver metabolism and circulating levels of TH (Flamant and Samarut, 2003).

The TRs and other nuclear receptors all share a common modular structure. Two functional domains are particularly important: the DNA-binding domain, which is involved in the recognition of short DNA sequences on target genes, and the ligand-binding domain (LBD) that can bind T3 with high affinity. Binding of T3 induces conformational changes of the TR LBD that modify its association with coregulator proteins involved in controlling the transcriptional activity of the receptors (Zhang and Lazar, 2000). The transcription of a large subset of TH target genes is up-regulated by T3-bound TRs (holo-TRs), whereas the transcription of other genes is down-regulated by holo-TRs. Furthermore, TRs are also transcriptionally active in the absence of T3 (apo-TRs), albeit in a manner opposite to the activity of the holo-TRs. This complex regulatory pattern depends on the interaction of the apo- and holo-TRs with transcriptional coregulator proteins.

Basal expression of positively T3-regulated genes is repressed by apo-TRs and stimulated by holo-TRs. Target gene silencing by apo-TRs is mediated by binding of corepressor proteins, such as the silencing mediator of the retinoid and thyroid hormone receptor (SMRT) or the nuclear receptor corepressor (NCoR), to the apo-TR LBD. These coregulators also allow the recruitment of histone deacetylases that maintain the promoters of target genes in a repressed state, and repress their basal expression (Eckey et al., 2003). Conversely, binding of T3 to the holo-TRs induces conformational changes that result in corepressor release and recruitment of coactivator proteins, such as those of the p160/SRC (steroid receptor coactivator) family, including SRC1, SRC2, SRC3. These coactivators possess constitutive histone acetylase activity and can recruit additional factors with histone acetyltransferase activity that are involved in enhancing the transcription of target genes by T3 (Xu and Li, 2003).

The regulation of negatively T3-regulated genes by coregulators seems to be the reverse of that of positively T3-regulated genes. Their basal expression is stimulated by apo-TRs, and repressed when T3 is added. Although the mechanisms are less well characterized, it has been reported that NCoR and SMRT activate rather than suppress the basal transcription of several of these target genes (Tagami et al., 1997). Coactivators, such as SRC1, also can play an apparently paradoxical role, since they enhance the T3-induced suppression of the transcription of the thyroid stimulating hormone α (TSHα) gene (Tagami et al., 1999).

Short, highly-conserved peptide sequences play a major role in the association of coactivator and corepressor proteins with the TR LBD, depending on the LBD conformation adopted in the presence or absence of T3. Coactivators contain several consensus LXXLL motifs (where L = leucine and X = any amino acid) in their receptor interaction domain, whereas the corepressors NCoR and SMRT contain several LXX I/H IXXX I/L motifs (where I = isoleucine and H = histidine). Both motifs bind to overlapping sites on the LBD, although the coactivator consensus sequence is preferentially bound by the holo-LBD, whereas the corepressor consensus motif shows high affinity for the apo-LBD (review in Aranda and Pascual (2001)). Therefore, short peptides containing these consensus sequences have been used to probe the LBD conformational state of several nuclear receptors, including TRs. An *in vitro* fluorescence polarization (FP) assay was used by Arnold et al. (2005) to measure the ability of small molecules to inhibit the interaction between the TRβ LBD and a SRC2 peptide. Binding of SRC1, SRC3, and NCoR fragments to the TRα1-LBD or TRβ-LBD have been quantified using the AlphaScreen technology (Koury et al., 2009). In the present study, we used FP to quantify binding of fluorophore-labeled SRC2 and NCoR peptides to bacterially-produced recombinant TRα1 LBDs. The cell-free assays were optimized and validated with T3 analogs and hormones with known thyromimetic properties.

3,3′,5,5′-Tetrabromobisphenol A (TBBPA) is widely used as a reactive or additive flame retardant in polymers, resins, adhesives, textiles, and in the manufacture of printed circuit boards. It is the most abundant brominated flame retardant, with a worldwide production volume of over 2×10^5 metric tonnes (Alaee et al., 2003). It is released into the environment from both additive- and reactive-treated products, and measurable amounts were detected in air, soils, and sediments (review in Birnbaum and Staskal (2004)). A recent extensive review (Covaci et al., 2009) points to significant levels of TBBPA measured in biological matrices from marine biota, including invertebrate, fish, birds and mammals, the most important concentrations being found at the top of the food chain. Concentrations of up to 35 μg kg^{-1} wet weight have been reported in porpoise blubber (Law et al., 2006) and up to 36 ng g^{-1} lipid in shark muscle (Johnson-Restrepo et al., 2008). The range of TBBPA concentrations measured in human body fluids is rather large (Sjodin et al., 2003; Covaci et al., 2009), which may depend on whether people were occupationally exposed at work, or lived in the vicinity of the sources of pollution. Thus, TBBPA was detected at levels ranging from 0.34 to 0.71 ng g^{-1} lipids in human plasma samples from Norway (Thomsen et al., 2002). In a French survey conducted on women hospitalized for caesarean deliveries, maximum values of 93, 37, and 650 ng TBBPA g^{-1} lipid were found in milk, maternal serum, and cord serum, respectively, pointing to significant exposure of both mothers and fetuses (Cariou et al., 2008).

It has been suggested that the primary toxic effect of TBBPA, as of other brominated flame retardants, is the disruption of thyroid homeostasis (Birnbaum and Staskal, 2004). TBBPA has been reported to bind to TRs *in vitro*, and to display an anti-TH effect in a mammalian cell model and on amphibian metamorphosis (Kitamura et al., 2005). Conversely, the same group reported a thyromimetic effect of TBBPA in a TH-responsive pituitary cell line (Kitamura et al., 2002).

Using TRα1-expressing cells and reporter gene technology, we have previously reported that several halogenated chemicals, including TBBPA, act both as partial agonists and as T3-antagonists on TRα1 (Jugan et al., 2007). The primary objective of the present study was to use validated cell-free coregulator peptide-binding assays to investigate the conformational changes of TRα1 LBD induced by TBBPA, either alone or in combination with T3. As a consequence, TBBPA might alter the transcription of genes that are up- or down-regulated by both the apo- or the holo-TRα1.

2. Materials and methods

2.1. Labeled peptides and other reagents

Peptide sequences of box 3 of the NCoR corepressor and of boxes 2 of the SRC2 coactivator were chosen because they have