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Novel cell-based assay for detection of thyroid receptor beta-interacting environmental contaminants

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Abstract

Even though the presence of endocrine disrupting chemicals (EDCs) with thyroid hormone (TH)-like activities in the environment is a major health concern, the methods for their efficient detection and monitoring are still limited. Here we describe a novel cell assay, based on the translocation of a green fluorescent protein (GFP) - tagged chimeric molecule of glucocorticoid receptor (GR) and the thyroid receptor beta (TR β) from the cytoplasm to the nucleus in the presence of TR ligands. Unlike the constitutively nuclear TR β , this GFP-GR-TR β chimera is cytoplasmic in the absence of hormone while translocating to the nucleus in a time- and concentration-dependent manner upon stimulation with triiodothyronine (T3) and thyroid hormone analogue, TRIAC, while the reverse triiodothyronine (3,3',5'-triiodothyronine, or rT3) was inactive. Moreover, GFP-GR-TR β chimera does not show any cross-reactivity with the GR-activating hormones, thus providing a clean system for the screening of TR beta -interacting EDCs. Using this assay, we demonstrated that Bisphenol A (BPA) and 3,3',5,5'-Tetrabromobisphenol (TBBPA) induced GFP-GR-TR β translocation at micro molar concentrations. We screened over 100 concentrated water samples from different geographic locations in the United States and detected a low, but reproducible contamination in 53 % of the samples. This system provides a novel high-throughput approach for screening for endocrine disrupting chemicals (EDCs) interacting with TR beta.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Keywords

EDCs; TR β ; high-throughput cell assay; BPA; TBBPA

1. Introduction

Thyroid hormones are critical for normal development, growth, and metabolism of all vertebrates, including mammals (Grimaldi et al. 2013; Lopez-Juarez et al. 2012; Pascual and Aranda 2013; Sirakov et al. 2013; Zoeller et al. 2002). They are involved in important physiological functions including, but not limited to neurogenesis and brain function (Horn and Heuer 2010; Reinehr 2010), homeostasis (Warner and Mittag 2012), thermo-regulation (Ribeiro 2008), cardiovascular health (Danzi and Klein 2012; Vargas et al. 2012), reproductive health (Krassas et al. 2010; Wagner et al. 2008), osmoregulation and renal function (Vargas et al. 2006; Vargas et al. 2012).

The predominant TH in the circulation is 3,3',5,5'-tetraiodothyronine (thyroxine, T₄), which is the precursor for the active T₃ (3,3',5-triiodothyronine) form of the hormone. The functions of TH are mediated by the interaction of T₃ with the thyroid hormone receptors (TR α and TR β). These TRs are present in most tissues and their expression begins early in development (Zoeller et al. 2002). They are localized in the cell nucleus and interact with specific DNA sequences called TH-response elements (TREs). TRs bind to TRE as heterodimers with retinoid X receptor (RXR) and recruit a number of cofactors such as corepressors and coactivators (Zhang and Lazar 2000). Unliganded TR/RXR complex recruits corepressor complex containing NCoR (nuclear receptor corepressor) or SMRT (silencing mediator of retinoid and thyroid hormone receptor), which exhibits histone deacetylase (HDAC) activity. In this state, the TR/RXR heterodimer represses transcription by restricting the accessibility of basal transcription factors to targeted promoters. In the presence of T₃, the corepressors are replaced by coactivator complexes, which contain histone acetylase (HAT) activity. A recent study demonstrated that in addition to the hormone-independent TR occupancy there is a significant hormone-induced TR recruitment to chromatin associated with chromatin remodeling and activated gene transcription in mouse liver tissue (Grontved et al. 2015). It was demonstrated that the T₃ regulated genes can also respond to other hormonal signals. Thus, the action of the TH is often described as 'permissive' hormone action, indicating that the TH effects at the cellular, tissue, and organismal levels provide a platform for other biological signals. However, this 'permissive' action of the TH is crucial for body development and homeostasis (Grimaldi et al. 2013; Konig and Moura Neto 2002; Oetting and Yen 2007; Warner and Mittag 2012). Thyroid signaling disruption could arise due to altered hormone production, transport and metabolism, as well as by disruption of the existing feed-back mechanisms, or untimely receptor activation/deactivation (Gilbert et al. 2012). In addition, mistiming of TH-modulated events may have permanent effects on neurodevelopment, whereas in adults changes in TH signaling are typically easily treatable by pharmaceuticals with no permanent deleterious effects (Murk et al. 2013).

A growing number of studies indicate that contamination of the environment with endocrine disrupting chemicals, including chemicals interfering with the thyroid signaling, may have deleterious health effects. According to the World Health Organization an endocrine disruptor as an “exogenous substance or a mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or (sub) populations” (WHO, 2011). The Scientific Statements of the Endocrine Society from 2009 and 2015 postulate that in addition to the EDCs’ effects on reproduction, breast development and cancer, prostate cancer, neuroendocrinology, obesity, and cardiovascular endocrinology, EDCs can also negatively impact thyroid metabolism (Diamanti-Kandarakis et al. 2009; Gore et al. 2015). Therefore, there is a rising need to develop novel approaches for identification of EDCs interfering with the thyroid hormone signaling [summarized in (Murk et al. 2013)].

Here we describe the development and implementation of a rapid high-throughput cell-based screening assay for detection of EDCs with thyroid hormone-like activities.

2. Materials and Methods

2.1. Chemicals

Bisphenol A (BPA), 3,3',5,5'-Tetrabromobisphenol A (TBBPA), 3,3',5-Triiodo-L-thyronine sodium salt powder (T3), triiodothyroacetic acid (TRIAC), reverse triiodothyronine (rT3), and thyroxine (T4) were purchased from Sigma and their purity was above 95% (catalog Nu: 239658-50G, 330396-100G, T6397, 51-24-1, 5817-39-0, and 51-48-9, respectively). The organic solvent DMSO (catalog Nu: D2650) was also purchased from Sigma.

2.2. Water samples collection

Environmental water samples were collected as part of ongoing U.S. Geological Survey (USGS) projects that were implemented to monitor the presence and effects of endocrine-disruptors and other contaminants of emerging concern. They were collected between 2010 and 2013 from different geographic locations in the United States (Supplemental Table 1) and included discrete grab water samples and samples collected via polar organic chemical integrative samplers (POCIS). Grab water samples were processed at the USGS, Leetown Science Center as described below.

2.3. POCIS samples

The POCIS membranes were shipped to the USGS, Columbia Environmental Research Center for analyte recovery. The procedures for preparing the POCIS samples for analysis deviated slightly from those described earlier (Alvarez et al. 2009). Briefly, chemicals were extracted from the POCIS sorbent using 25 mL of 80:20 (V:V) dichloromethane:methyl-*tert*-butyl-ether. The extracts were reduced by rotary evaporation, filtered, and composited into 2-POCIS equivalent samples thereby concentrating the amount of chemical present in each sample to aid in the detection.

2.4. Grab Water samples

Grab water samples were collected in 1 L pre-cleaned amber glass bottles with Teflon lined caps (C&G Containers Scientific Supplies, Lafayette, LA). Water was acidified to pH3, held on ice, and stored at 4 °C. Within one week of collection, the preserved water samples were filtered through a GF/F filter (0.7 µm) using a solvent rinsed all-glass apparatus. Filters were rinsed with 1 ml of methanol to liberate soluble compounds from the retained suspended solids. Filtered samples and blanks were subjected to solid phase extraction (SPE) using OASIS® HLB (200 mg) glass cartridges (Waters Corporation, Milford, MA), following an existing protocol (Ciparis et al. 2012). In short, cartridges were sequentially pre-conditioned and 800 ml of filtered samples were loaded onto the cartridge at a flow rate of 5–6 ml/minute (continuous vacuum). Analytes were eluted from the cartridge with 100% methanol and concentrated by evaporation. For biological testing, samples were reconstituted in DMSO and diluted in growth media to a final 1,000x concentration from the original water volume while maintaining DMSO at <0.2%. Samples were added to cells for 3h at 100x concentration or as indicated in the text.

2.5. Construction of the GFP-GR-TRβ chimeric receptors

GFP-GR-TR TRβ construct was generated by fusing the human GR N-terminus, DNA binding domain (DBD) and hinge regions upstream of a hybrid ligand binding domain (LBD) composed of hGR helix 1 and partial loop 1–3 sequences linked to portions of the hTRβ LBD. The eGFP-GR-TR216 chimera containing eGFP-linker-hGR (2-552)-hTR LBD (216-end) and the eGFP-GR-TR226 chimera containing eGFP-linker-hGR (2-552)-hTR LBD (226-end) were prepared in spectinomycin resistant GATEway (Thermo Fisher Scientific, Waltham, MA) Entry clones. All entry clones were sequenced throughout the entire cloned region and were found to completely match the expected DNA sequence. Ampicillin resistant pFUGW lentiviral vectors expressing clones were generated with hygromycin selection marker and a Tet-regulated pTRE-tight promoter. Transfection-ready DNA was prepared using the Sigma GenElute XP Maxiprep kit and verified by agarose gel electrophoresis and restriction digest.

2.6. Cell line development

The MCF7 Tet-off Advanced cell line (Clontech, Mountain View, CA) was maintained in DMEM media supplemented with 10% calf serum, 2 mM glutamine, and penicillin/streptomycin. Transient transfections were performed following the manufacturer's protocol using Lipofectamine 2000 reagents. For the development of stable cell line, MCF7 Tet-off Advanced cells were first infected with lentivirus containing the eGFP-GR- TRβ 226 11.6 kB ampicillin resistant pFUGW lentiviral expression clone with a tet-regulated pTRE-Tight promoter and 48 hours later challenged with hygromycin to generate polyclonal cell lineage. Single cell clones were generated by limiting dilution and tested for eGFP-GR- TRβ 226 expression and response to T3 treatment.

2.7. Cell treatment and imaging

Prior to imaging, cells were grown for 24 hours in DMEM medium (Dulbecco's Modified Eagle's Medium, Gibco) containing 10% charcoal stripped serum (Hyclone, Logan, UT)

without tetracycline (to allow the expression of the GFP-GR-TR). Then the cells were plated in 96 well plates (Matrical, Catalog Number MGB096-1-2-LG-L) at a density 30, 000 cells per well (again in DMEM medium containing 10% charcoal stripped serum without tetracycline) and grown at 37°C for additional 24 hours. Cells were treated with vehicle control, or various concentrations of T3, BPA, and TBBPA for 3 hours. For the time-response experiment cells were treated with 100 nM T3 for various lengths of time before fixation. Water sample extracts were applied for 3 hours at 37°C at a final concentration of 100x, if not specified otherwise. Additional negative controls contained samples that tested the activity of the POCIS membranes themselves. Upon treatment, cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed 3x with PBS. Cells were further stained with DRAQ5 (BioStatus Limited) at concentration 1:5000 for 10 minutes and after 3 final washes with PBS were imaged either immediately on the Perkin Elmer Opera Image Screening System or kept in PBS at 4°C for later imaging.

2.8. Automated imaging and analysis by Perkin Elmer Opera Image Screening System

A PerkinElmer (Waltham, MA) Opera QEHS High-Content Screening platform was used for fully automated confocal collection of images. This system employed a 40x water immersion objective lens, laser illuminated Nipkow disk, and cooled CCD cameras to digitally capture high-resolution confocal fluorescence micrographs (300 nm pixel size with 2x2 camera pixel binning). An image analysis pipeline was customized using the Columbus software (PerkinElmer) to automatically segment the nucleus using the DRAQ5 channel and then construct a ring region (cytoplasm) around the nucleus mask for each cell in the digital micrographs. The pipeline filtered only for cells expressing GFP-GR- TR β out the cells then measured the mean GFP-GR- TR β intensity in both compartments in the GFP channel, and translocation was calculated as a ratio of these intensities. Each value was further normalized to the value for the control (DMSO) sample on the same plate.

2.9. Gene transcription analysis

For gene transcription studies, TR β 1 expressing HepG2 cells (Chan and Privalsky 2009) were plated in 24-well dishes 24 hours before experiment in DMEM media supplemented with charcoal stripped fetal bovine serum (Hyclone, Logan, UT). Cells were treated with water samples, vehicle control (DMSO) or T3 hormone for 30 minutes. To prevent cellular stress, these experiments were performed in a specially adapted incubator, allowing treatment under conditions of stable CO₂ and temperature levels throughout the duration of an experiment. Cells were lysed in 600 μ l of RLT buffer (with β -mercaptoethanol added) followed by syringe/needle shearing. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), including a DNaseI digestion step (RNase free DNase Set, Qiagen). One μ g of RNA was reverse transcribed (iScript cDNA Synthesis Kit, BioRad) in 20 μ l reaction volume and 0.5 μ l was used per Q-PCR reaction using SyBr green and Bio-Rad IQ system (Biorad, Hercules, CA). Primer sequences for Phosphoenolpyruvate carboxykinase 1 (hPCK1) and Coenzyme Q10 homolog A (COQ10A) were designed to amplify nascent RNA (amplicons that cross an exon/intron boundary). The primer sequences are shown below. PCR was performed as recommended by a manufacturer. Standard curves were created by 4-fold serial dilution of template. The expression data from three or more independent experiments were normalized to the expression of a control gene GAPDH. The

mean values and SEM were calculated and displayed as a fold change in relation to the control (DMSO treated) sample. Primer sequences for Q-PCR analysis are as follows:
 hPCK1 For: 5'-TGACAGTGGGCTGTTTGTTC-3', hPCK1 Rev: 5'-
 AAGACCCCAAGTGCCTTTCT-3', hCOQ10A For: 5'-
 TTTCAAGGATGCTGGCTCTT-3', hCOQ10A Rev: 5'-
 GGCCTCAGCTTGTCAAATTC-3', hGAPDH For: 5'-
 AAGGTGAAGGTCGGAGTCAAC-3', hGAPDH Rev: 5'-
 GGGGTCATTGATGGCAACAATA-3'.

2.10. Statistical Analyses

Data were analyzed using the graphical and statistical functions of SigmaPlot 11 (SPSS Inc., Chicago, IL). All experiments were repeated at least 3 times; the mean value was calculated for each sample. The mean values were used in a one-way analysis of variance test. If a significant F-value of $P < 0.05$ was obtained, a Holm-Sidak's multiple comparison versus the control group analysis was conducted.

2.11. Method for calculation of T3 equivalent (T3-EQ) activities in the water samples

To determine the T3- equivalent (T3-EQ) levels when examining unknown EDCs we first used a calibration dose-response curve for the GFP-GR- TR β translocation in response to T3 (vehicle treated control, 0.39 nM, 0.78 nM, 1.56 nM, 3.13 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM). To achieve a better standardization and reduce the errors due to some inherent variability in the experimental values for nuclear translocation we further normalized the data so that the translocation value of the control condition equals 0 and translocation value upon 3-hours treatment with 100 nM T3 equals 1. The resulting curve was plotted and a fit was performed using a formula $y = \tanh(a_0 \cdot x^{a_1})$, where $a_0 = 0.25112$ (± 0.03) and $a_1 = 0.59015$ (± 0.06). To calculate T3-EQ of any particular sample, its translocation value was calculated in respect to the negative and positive control translocation values (vehicle treated cells and 100 nM T3-treated cells, respectively) obtained for this particular experiment. The resulting value was then used to calculate the T3-EQ using the formula $x = (\tanh(y)/a_0)^{1/a_1}$, where x is the T3-EQ. This approach reduces the experiment-to-experiment variability arising from fluctuations in the GFP-GR-TR β expression levels.

3. Results

3.1. Construction of chimeric receptor responsive to T3 and generation of a cell line stably expressing the chimera

Two chimeras containing a GFP reporter and human glucocorticoid receptor fragment (2-552 aa) fused to thyroid receptor beta ligand binding domain (LBD) were constructed in lentiviral vectors (Fig. 1A). Even though the two chimeric receptor constructs differed by 10 amino acids, only the chimera containing hTR β LBD (216aa-end and not the 226aa-end) translocated to the nucleus upon treatment with 100 nM T3 (data not shown). Therefore, only this construct was further used in MCF7 Tet-off Advanced cells to establish a stable cell line, depicted schematically in Fig. 1B. These cells are only responsive to the T3 stimulation while a treatment with the GR activating hormone dexamethasone did not induce

GFP-GR-TR β translocation indicative of specific response to TH (Fig. 1C and Supplementary Fig. S1).

3.2. Concentration- and time-dependent response of the GFP-GR-TR β chimera to T3

To characterize the newly developed cell line and establish the optimal treatment time for the assay we induced the cells with 100 nM T3 for 1, 2, 3 and 4 hours and compared the level of the GFP-GR-TR β translocation under those conditions to the control (unstimulated) cells. We quantified the GFP-GR-TR β translocation by implementing the automated Perkin Elmer Opera Image Screening System and using an algorithm for cytoplasm and nuclear segmentation to calculate translocation efficiency (Stavreva et al. 2012). We found that GFP-GR-TR β translocation was time-dependent (Fig. 2A). Significant translocation was observed even after 1h of incubation with T3. However, the response at 3h and 4h was significantly higher with over 2-fold change. To determine the sensitivity of the assay, we treated cells with increasing concentrations of T3 incubated with cells for 1, 3 or 4 h (Fig. 2B). We observed concentration-dependent increase of the GFP-GR-TR β translocation in all three cases. However, the response at 1h plateaued at much lower translocation levels while the 3h and 4h treatments showed similar concentration dependent responses. We concluded that a 3h treatment was optimal for this assay (Fig. 2C).

3.3. GFP-GR-TR β chimera translocates in response to TRIAC but not to rT3

We then examined the response of the GFP-GR-TR β chimera to increasing concentrations of the thyroid hormone analogue TRIAC (also known as Tiratricol or triiodothyroacetic acid) and found that it was similar to the response elicited by T3 (Fig. 3A and B). In contrast, a treatment with the prohormone thyroxine (T4) induced a much attenuated receptor translocation, consistent with the fact that T4 requires enzymatic conversion to T3. As expected, the reverse triiodothyronine (3,3',5'-triiodothyronine, or rT3), which is an isomer of T3, was largely inactive (Fig. 3A and B).

3.4. GFP-GR-TR β chimera translocates in response to BPA and TBBPA

To test the translocation response of GFP-GR-TR β chimera to known endocrine disruptors, we treated the cells with Bisphenol A (BPA) and Tetrabromobisphenol A (TBBPA), a halogenated derivative of BPA. Both chemicals are highly abundant environmental contaminants. BPA is employed to make certain plastics and epoxy resins, while the TBBPA is a commonly used fire retardant. It was previously demonstrated that BPA and its halogenated derivatives can inhibit T3 binding to TRs in μ M concentrations (Freitas et al. 2011; Moriyama et al. 2002; Sun et al. 2009). Consistent with these reports, we observed concentration-dependent translocation of the GFP-GR-TR β chimera in response to μ M concentrations of BPA and TBBPA (Fig. 4 A and B). We concluded that our assay is capable of detecting the presence of BPA and its derivatives.

The GFP-GR-TR β chimera is highly sensitive and detects a wide range of T3 concentrations (1.6 to 100 nM) which covers both the physiological and pharmacological hormonal range (Fig. 2 and 3). We thus tested the effect of the combined treatment using low physiologic doses of T3 (5 nM) and low dose of BPA that elicited a measurable activity (25 μ M). We

found that this combination induced a significantly higher translocation than the individual compounds, suggesting an additive effect (Fig. 4C).

3.5. Screening water samples for TR β -interacting contaminants

Next, we implemented the GFP-GR-TR β expressing cells to test over 100 concentrated water samples collected at the sites in US indicated in Fig. 5. We discovered a low but reproducible activity in 53% of the samples (see also Supplementary Fig. S2). These samples were tested at 100X concentration, indicating low level of contamination capable of activating TR β . We further tested the effect of three of these water samples that tested positive for translocation and one negative sample (8, 13, 15 and 9, respectively) on transcriptional activity of two TR β -regulated genes: PCK1 and COQ10A in HepG2 cells. As shown in Fig. 6 (A and B), both genes were induced in the presence of T3, and their response was time- and concentration-dependent. As shown in Fig. 6C and D, positive water samples increased transcription of PCK1, but either did not affect the transcription of COQ10A (sample 8) or reduced the basal levels of expression (samples 13 and 15, respectively), suggesting that the contaminant mixtures in these water samples may elicit complex and gene-specific patterns.

3.6. T3 equivalent (T3-EQ) activities in the water samples

To compare the level of biological activity, present in the collected water samples to the translocation activity elicited by known levels of T3, we first generated a T3/translocation dose-response curve (Fig. 7A). This curve was further used as a standard to calculate the T3 equivalent (T3-EQ) activities of the samples (Material and Methods section). The calculated T3-EQ activities of the water samples ranged from 0 nM T3 (negative samples, data not shown) to over 1.5 nM T3 (Fig. 7B). We also calculated the T3-EQ for the samples treated with BPA and TBBPA. As shown in Fig. 7C the obtained T3-EQ values for the highest tested dose (100 μ M) were just 0.9 nM and 5.9 nM for BPA and TBBPA, respectively. Considering the prevalent contamination of the environment with BPA worldwide (Corrales et al. 2015) it is plausible that BPA could be also present in some of the tested water samples. Indeed, an earlier study found 12 μ g/L BPA in effluents in the US water (Kolpin et al. 2002).

4. Discussion

Contamination of the environment with chemicals with thyroid-disrupting properties is a major health concern as thyroid hormone receptors are involved in many aspects of physiology and development. Several approaches and cell lines for detection of EDCs, including tests for thyroid hormone disruption (Fini et al. 2007; Grimaldi et al. 2015; Scholz et al. 2013; Simon et al. 2010; Steinberg 2013) have been proposed. However, alternative methods for fast and low-cost high-throughput detection of TR-interacting EDCs in the environment are still in a great demand.

We have previously reported a mammalian cell-based screening method for detection of biologically active glucocorticoids and androgens in environmental samples (Stavreva et al. 2012). By tagging glucocorticoid and androgen receptors with a fluorescent protein such as GFP, we can follow cellular localization of these receptors from the cytoplasm to the nucleus

in response to natural and synthetic ligands as well as compounds with hormone-like activities. This assay is compatible with a high-throughput, high-content imaging analysis which automatically detects and quantifies the intensity of the GFP-tagged nuclear receptor in both the cytoplasm and in the cell nucleus. An algorithm for nuclear segmentation is then used to calculate efficiency of the receptor translocation indicative of the presence of hormonal activity interacting with the hormone binding domain of the receptor (Stavreva et al. 2012). Here we describe the development and implementation of a novel assay for detection of TR β -interacting contaminants based on the same nuclear translocation principle. However, unlike GR (Htun et al. 1996; Ogawa et al. 1995) or AR (Klokk et al. 2007), the thyroid receptor isoforms are mostly nuclear even in the absence of hormone (Baumann et al. 2001) and thus cannot be used directly in a nuclear translocation assay. To overcome this obstacle, we generated a fusion GFP-GR-TR β construct, an approach used earlier to generate fluorescent chimeric molecules between the GR and the retinoic acid receptor (GFP-GR-RAR) and the GR and estrogen receptor (GFP-GR-ER) (Mackem et al. 2001; Martinez et al. 2005). The two major isoforms of the human thyroid receptor TR α and TR β are encoded by two separate genes *THRA* and *THRB* located on chromosomes 3 and 17, respectively. Alternative splicing gives rise to additional isoforms: four thyroid hormone binding isoforms (β 1, β 2, β 3 and α 1) and two TR α truncated isoforms that do not bind T3 (α 2 and α 3). The C-terminal domains of the T3-binding TRs are highly homologous. The ligand binding domain (LBD) of all TR β isoforms are identical and show over 80% sequence similarity with the TR α (Cheng 2005). Therefore, we decided to use the C-terminal LBD of human TR β for generation of the GFP-GR-TR β chimera. Previous studies demonstrated similar affinities of TR α and TR β to the physiologic ligand, T3. However, other synthetic ligands are reported to elicit differential responses (de Araujo et al. 2010). Thus, the results our studies are mainly applicable to TR β . Importantly, unlike the WT receptor the GFP-GR-TR β chimeric molecule resides in the cytoplasm in its non-induced state and translocates to the nucleus in response to T3 stimulation. We observed a concentration- and time-dependent GFP-GR-TR β translocation in response to T3 and TRIAC without any cross-reactivity with GR-activating hormone (Supplementary Fig. S1), thus providing a clean system for the screening of TR β -interacting EDCs. Because the prohormone T4 has low affinity and requires enzymatic conversion to T3 to gain TR binding capability, GFP-GR-TR β translocation in response to T4 was greatly reduced (Fig. 3). Finally, a treatment with rT3, the inactive isomer of T3, failed to induce GFP-GR-TR β translocation (Fig. 3) demonstrating that the chimera detects only biologically active TR hormones and analogs.

Treatments with BPA and TBBPA, representing previously described EDCs with known TR activity, induced GFP-GR-TR β translocation in a concentration-dependent manner. Interestingly, it was shown that halogenated BPAs are more structurally similar to TH than BPA and it was reported that TBBPA is active at lower concentrations than BPA (Fini et al. 2007). Our experiments corroborate these findings, as TBBPA treatment induced higher levels of GFP-GR-TR β translocation than BPA at all tested concentrations. We also tested over 100 concentrated environmental water samples and discovered that 53% of them were able to induce partial GFP-GR-TR β translocation indicative of a low, but prevalent contamination of water samples with TR β -interacting contaminants. It should be noted that

our model system cannot be applied to study TR β -mediated gene transcription because the respective TR β DBD is missing from this chimeric construct. This system functions purely as a sensor for TR β -interacting chemicals in the cellular milieu without discrimination between agonist or antagonistic chemicals and is intended as an initial high-throughput screen. It could be followed by chemical analyses and transcriptional studies using endogenous genes or luciferase based reporter constructs. TR beta CALUX reporter gene assay, for example, is a high-throughput screening method based on TR β -mediated transcriptional response (Simon et al. 2010; Steinberg 2013). Both, the TR β CALUX and the translocation assay show activity in the picomolar and nanomolar range upon stimulation with T3. However, the translocation assay has a clear advantage as it is much faster (requires only 3 hours), thus saving time and minimizing the chance for nonspecific toxic effects. Another major advantage of the translocation assay is that it detects both agonists and antagonists, as well as mixtures containing the two activities which makes it a broader screening tool. For example, if a sample contains both agonists and antagonists it will be highly positive in the translocation assay, but may elicit only a weak response or no response at all in the transcriptional assay, as the two activates may cancel each other according to their concentrations. Indeed, we studied transcriptional activity of a number of translocation-positive samples using HepG2 cells which express TR β 1 (Chan and Privalsky 2009) and demonstrated a complex and gene-specific pattern of gene regulation that could reflect the presence of a mixture of TR β -interacting chemicals. Previously published studies using TR-mediated luciferase gene expression assay detected T3-like activity in water and effluents from water treatment plants (WWTP) in Japan (Murata and Yamauchi 2008) and anti-T3 hormonal activity in WWTP effluent in Thailand (Ishihara et al. 2009), demonstrating that both agonists and antagonists could be present in the environment. Our data are the first report demonstrating the presence of TR β -interacting contaminants in US water sources. However, further analysis by chemical approaches will be required to determine the nature of these contaminants.

Considering that the samples were highly concentrated (100x) it might be tempting to speculate that the net biological impacts of the detected activities might be negligible. Indeed, when we recalculated the T3 equivalent (T3-EQ) activities of the samples, the highest T3-EQ was less than 2 nM (Fig. 7B). These results indicate a low level of TR β -interacting contaminants, but could also indicate a presence of chemicals like BPA or BPA derivatives, which have a low translocation-potency. Considering that even low levels of EDCs may have substantial consequences during development, this water contamination should not be overlooked and we suggest that its possible impact should be studied further.

5. Conclusions

Many of the EDCs exert their effects through direct interaction with endocrine hormone receptors and these interactions are the basis of an assay we recently developed to screen for glucocorticoid and androgen contaminants in water sources (Stavreva et al. 2012). Using the same principle, we now generated a cell line expressing GFP-tagged chimera between glucocorticoid and thyroid receptor beta (GFP-GR-TR β) that was successfully used to detect known TR β -interacting EDCs, as well as to screen novel chemicals with unknown EDCs potential, or concentrated water samples for thyroid hormone-like activities. This approach

is applicable to other nuclear receptors that could be designed by genetically engineered fusion with GR to become nuclear and translocate only in response to their respective ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EDCs	endocrine disrupting chemicals
TH	thyroid hormone
GR	glucocorticoid receptor
TR	thyroid receptor
T3	3,3',5-triiodothyronine
TRIAC	Tiratricol or triiodothyroacetic acid
rT3	reverse triiodothyronine or 3,3',5'-triiodothyronine
T4	thyroxine
BPA	Bisphenol A
TBBPA	3,3',5,5'-Tetrabromobisphenol A
TREs	TH-response elements
RXR	retinoid X receptor
NCoR	nuclear corepressor
SMRT	silencing mediator of retinoid and thyroid hormone receptor
HDAC	histone deacetylase
HAT	histone acetyl transferase
POCIS	polar organic chemical integrative samplers
DBD	DNA binding domain

LBD	ligand binding domain
hPCK1	Phosphoenolpyruvate carboxykinase 1
COQ10A	Coenzyme Q10 homolog A
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
T3-EQ	T3-equivalent

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Figure 1. Generation of a cell line expressing GFP-GR-TR β chimeric receptor

(A) Graphical representation of the strategy used for generating GFP-GR-TR β chimeric receptors. Only the GFP-GR-TR β 216 was capable of translocating from the cytoplasm to the nucleus in response to T3 and was used to create a cell line stably expressing the chimera in a tet-off manner. (B) Schematic representation of GFP-GR-TR β translocation in response to hormonal treatment. (C) GFP-GR-TR β translocates from the cytoplasm to the nucleus of the mammalian cell line (MCF7) upon stimulation with 100 nM T3 for 3h, while control (DMSO) treatment or treatment with the GR ligand dexamethasone failed to induce GFP-GR-TR β translocation (see also Supplemental Fig. S1). Nuclei are stained with DRAQ5. Scale bar, 10 μ m.

Figure 2. Time- and concentration-dependent nuclear translocation of GFP-GR-TR β in response to T3

(A) Time-dependent GFP-GR-TR β translocation in response to 100 nM T3 as detected by the Opera (Perkin Elmer) automated imaging analysis system. An algorithm for cytoplasm and nuclear segmentation of the cells was used to determine the mean GFP-GR-TR β intensity in both compartments and translocation was quantified as a ratio of these intensities. Each value was normalized to the control sample. Error bars represent the mean value $\pm$ s.e.m, $n=4$ ($P<0.05$, asterisks). (B) GFP-GR-TR β nuclear translocation in response to varying concentrations of T3 over 1h, 3h and 4h incubation. Error bars represent the mean value $\pm$ s.e.m, $n=6-12$. (C) Representative images of the GFP-GR-TR β nuclear translocation upon treatment with different concentrations of T3 for 3h. Nuclei are stained with DRAQ5. Scale bar, 10 μ m.

Figure 3. GFP-GR-TR β translocation in response TRIAC, T4 and rT3

(A) Concentration-dependent GFP-GR-TR β translocation upon treatment with TRIAC, T4 and rT3 for 3h at 37°C. A treatment with T3 is included as a positive control. Error bars represent the mean value $\pm$ s.e.m, n=8. (B) Representative images of the GFP-GR-TR β nuclear translocation upon 3-hour treatment with 125 nM of the indicated drugs or the corresponding DMSO control. Nuclei are stained with DRAQ5. Scale bar, 10 μ m.

Figure 4. Nuclear translocation of GFP-GR-TR β in response BPA and TBBPA

(A) Both BPA and TBBPA induced significant nuclear translocation of the GFP-GR-TR β chimeric receptor at all 3 tested concentrations ($P < 0.05$) upon 3h incubation at 37°C. The GFP-GR-TR β translocation to 100 nM T3 under identical treatment conditions is shown in a separate graph. Error bars represent the mean value $\pm$ s.e.m, $n=7$. (B) Representative images of the GFP-GR-TR β translocation upon treatment with 100 μ M BPA or TBBPA for 3h. Nuclei are stained with DRAQ5. Scale bar, 10 μ m. (C) A combined treatment of 25 μ M BPA and 5 nM T3 for 3h induced a heightened GFP-GR-TR β translocation response, indicative of an additive effect of the two stimuli. All treatments were significantly different from the control (DMSO), and the combined BPA+T3 treatment was significantly different from both T3 and BPA ($P < 0.05$). Error bars represent the mean value $\pm$ s.e.m, $n=4$.

Figure 5. Geographic locations of the collection sites and their contamination with TR β -interacting chemicals

Negative samples are marked with green color. Samples inducing significant GFP-GR-TR β translocation above the control are marked with red. For complete sample description (collection site, collection method as well as sample translocation activity) see Supplementary Tables S1.

Figure 6. Transcriptional effects of samples positive for GFP-GR-TR β translocation

(A) Levels of nascent RNA released from two TR β -regulated genes, PCK1 and COQ10A, upon treatment with 100 nM T3 reaches a plateau after 30 min of stimulation. Error bars represent the mean $\pm$ s.e.m., n=3. (B) Concentration-response of PCK1 and COQ10A gene transcription upon 30 minutes stimulation with T3. (C) Transcription of PCK1 is induced by 30 min-treatment with samples positive for GFP-GR-TR β translocation (8, 13, and 15) but not from a sample No. 9, which is negative for GFP-GR-TR β translocation (see also Supplemental Fig. S2). (D) Two of the three samples positive for GFP-GR-TR β translocation (13 and 15) inhibit the basal level of the COQ10A gene transcription, suggesting a gene-specific effects of the contaminants present in the samples. Error bars represent the mean $\pm$ s.e.m., n=3.

Figure 7. Calculation of T3 equivalent (T3-EQ) activities in the water samples positive for GFP-GR-TR β translocation

(A) The GFP-GR-TR β translocation values for known concentrations of T3 were renormalized from 0 to 1 (see Materials and Methods Section) and fitted using the formula $y = \tanh(a_0 \cdot x^{a_1})$, where $a_0 = 0.25112 (\pm 0.03)$ and $a_1 = 0.59015 (\pm 0.06)$. (B) T3 equivalent (T3-EQ) activities in the water samples positive for GFP-GR-TR β translocation were calculated using the formula $x = (\tanh(y)/a_0)^{1/a_1}$. (C) T3 equivalent (T3-EQ) activities in samples treated with BPA or TBBPA were also determined.