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4-Hydroxy-PCB106 acts as a direct thyroid hormone receptor agonist in rat GH3 cells

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Abstract

Polychlorinated biphenyls (PCBs) may interfere with thyroid hormone (TH) action by interacting directly with the TH receptor (TR). We found that the hydroxylated PCB metabolite, 4-OH-CB106, bound to the human TRβ1 and significantly elevated endogenous growth hormone (GH) expression in GH3 cells in a manner similar to that of T_3 itself. This effect was also observed using a consensus TH response element (TRE) in a luciferase expression system, and was blocked by a single base-pair substitution in this TRE. In addition, we found that 4-OH-CB106 did not alter the ability of TRβ1 to physically interact with the TRE in the GH promoter, or with SRC1 or NCoR. These effects were directly parallel to effects of T_3, indicating that 4-OH-CB106 exerts a direct agonistic effect on the TRβ1.
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Keywords: PCBs; Thyroid hormone receptors; Chromatin immunoprecipitation; GH3 cells; Brain development

1. Introduction

Polychlorinated biphenyls (PCBs) are industrial chemicals consisting of paired phenyl rings with various degrees of chlorination (Chana et al., 2002), producing 209 different parent congeners (Erickson, 2001). Although the production of PCBs was banned in the U.S. in 1979 and body burdens of PCBs are declining, these chemicals are routinely detected in the environment and in human tissues at significant concentrations (Breivik et al., 2002; Sjodin et al., 2004). For example, Sjodin et al. (2004) recently reported that serum levels of PCBs are in decline, and that polybrominated diphenyl ethers (PBDEs) are on the rise, but the median concentration of a single PCB congener (PCB 153) was still over half of the sum of all PBDE congeners measured. Thus, PCBs remain highly significant contaminants in the human population.

Epidemiological studies find that developmental exposure to PCBs is associated with neuropsychological deficits such as lower full-scale IQ, reduced visual recognition memory, attention deficits, and motor deficits (Ayotte et al., 2003; Huisman et al., 1995; Jackson et al., 1997; Korrick and Altshul, 1998; Osius et al., 1999; Walkowiak et al., 2001). Moreover, several studies find that PCBs, or specific PCB congeners, in maternal and cord blood are associated with lower thyroid hormone (TH) levels in both the mother and infant (Koopman-Esseboom et al., 1994; Schantz et al., 2003). Wang et al. (2005) reported that PCB levels in cord blood are negatively associated with free thyroxine (T_4) and with the product of free $T_4 \times$ TSH (as a measure of impacts on the negative feedback system). Although several studies report no association between PCB body burden and circulating levels of TH (Hagmar et al., 2001; Longnecker et al., 2000; Matsuura and Konishi, 1990; Sala et al., 2001; Steuerwald et al., 2000), experimental studies uniformly find that PCB exposure decreases circulating levels of T_4 in rats (Bastomsky, 1974; Bastomsky et al., 1976; Brouwer et al., 1998). Accordingly, a prevailing view is that PCB exposure may interfere with TH signaling by causing a relative state of hypothyroidism.

Abbreviations: PCB, polychlorinated biphenyls; TH, thyroid hormone; T_4, thyroxine; T_3, triiodothronine; Triac, triiodothyroacetic acid; Tetrac, tetraiodothyroacetic acid; TR, thyroid hormone receptor; TRE, thyroid hormone response element; ChIP, chromatin immunoprecipitation; SRC1, steroid receptor coactivator 1; NCoR, nuclear receptor corepressor; GH, growth hormone; CBP, cAMP response element binding protein-binding protein; ANOVA, one-factor analysis of variance

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However, experimental studies do not uniformly support the hypothesis that PCB exposure injures brain development by causing a relative state of TH insufficiency. For example, PCB exposure causes a severe reduction in circulating levels of T_4, but PCB exposed pups do not exhibit reduced body weight or body weight gain (Gauger et al., 2004; Sharlin et al., 2006; Zoeller et al., 2000), they exhibit elevated levels of expression of several TH-responsive genes in the brain (Bansal et al., 2005; Zoeller et al., 2000) and they do not always exhibit elevated serum TSH levels (Goldey et al., 1995). Thus, it is not resolved whether PCBs interfere with TH action in tissues by causing a state of relative TH insufficiency, or by exerting direct actions of PCBs on TH signaling.

PCBs can be hydroxylated *in vivo*, forming compounds that are prevalent in human and animal tissues, and which bind to the TH receptor (TR) (Cheek et al., 1999). Therefore, PCB metabolites may exert direct actions on TH signaling. Cheek et al. (1999) first published that 4-OH-CB106 binds to human TRβ1, although at low affinity. This observation led several other groups to begin to evaluate the ability of this and other PCB congeners and metabolites to bind to the TR and to affect gene expression (Zoeller, 2005). These studies present paradoxical findings in that some investigators find that PCBs act as TR agonists (Kitamura et al., 2005), while others report that PCBs act as TR antagonists (Miyazaki et al., 2004). Some of these discrepancies may be due to the endpoints used to evaluate the functional effect of PCBs on TR actions. For example, Kitamura et al. (2005) evaluated the effects of PCBs on growth hormone (GH) production and cell proliferation in GH3 cells, whereas, Miyazaki et al. (2004) used a luciferase expression system to characterize the functional effects of PCBs on transcriptional regulation by TR. Considering these discrepancies, we used a variety of approaches to characterize the functional effects of 4-OH-CB106 on TH signaling in GH3 cells, including effects on endogenous GH mRNA levels, luciferase activity in a reporter system, and the ability of the TR to associate with the GH promoter using chromatin immunoprecipitation (ChIP).

2. Materials and methods

2.1. TR binding assay

For saturation analysis, 40 ng recombinant hTRβ1 (Active Motif, Carlsbad, CA) was incubated in assay buffer [20 mM Tris–HCl (pH 7.6), 1.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.1% BSA] with increasing concentrations of ^{125}I-T_3 (1.5×10^{-7} to 8×10^{-9} M; 3300 μCi/μg, NEN, Boston, MA) in a final volume of 300 μl. Reactions were incubated at 37 °C for 30 min. Non-specific binding was determined in parallel tubes containing no TR. The reaction was terminated and bound counts were separated from free by incubating each reaction with a 25%-slurry of AG1-X8 resin (1:1, v/v, in assay buffer, Bio-Rad) (Samuels et al., 1979) and vortexing for 5 min at room temperature. Samples were centrifuged at $1500 \times g$ for 5 min and the supernatant was removed to a 12 mm × 75 mm test tube and counted in Cobra II gamma counter (Packard Instruments, Downers Grove, IL). Based on saturation analysis, 3×10^{-10} M ^{125}I-T_3 was used in competitive binding experiments with increasing concentrations of competitors. Non-specific binding was determined as described for saturation analysis.

2.2. Cell culture

GH3 cells were obtained from the American Type Tissue Collection (ATTC, Rockville, MD) and were maintained in Hams F-12K media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Mediatech, Herndon, VA), and 10% fetal bovine serum (FBS) (Hyclone, Sout Logan, UT) in a 37 °C humidified incubator with 5% CO_2.

Table 1
Comparison of the DR4 TRE sequence with the ΔDR4 TRE sequence[a]

TRE	5′ → 3′ Sequence
DR4	TTAT**AGGTCA**CATG**AGGTCA**AGTT
ΔDR4	TTAT**AGATCA**CATG**AGGTCA**AGTT

[a] Consensus half sites are presented in bold. The single base-pair mutation (G → A) is underlined.

2.3. Transient transfection assay

GH3 cells at 70% confluency were plated at a density of 2×10^5 cells per well in 24-well plates 24 h prior to transfections. Transfections were carried out using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Either the DR4 TRE (DR4-tk-Luc, Table 1) firefly luciferase vector, a 250 bp region of native rat GH TRE (bases −237 to +11 from the transcription start site, GH250-tk-Luc) firefly luciferase vector [both kindly provided by Dr. Douglas Darling (Cabanillas et al., 2001)], or a mutant DR4 TRE (ΔDR4-tk-Luc, Table 1, provided by Dr. Tony Hollenberg) firefly luciferase vector were co-transfected with the pRL-CMV renilla luciferase vector (Promega, Madison, WI) to control for transfection efficiency. Twenty-four hours after transfection, the media was removed and cells washed with 1× phosphate buffered saline (PBS), then fed with media containing hormone-stripped fetal bovine serum [FBS, stripped using 10% AG 1-X8 resin (Analytical grade; Bio-Rad, Hurcules, CA)] (Samuels et al., 1979). Cells were then treated with 0.1 μM T_3 (Sigma, St. Louis, MO) and/or 10 μM 4-OH-CB106 (AccuStandard Inc., New Heaven, CT) dissolved in EtOH or dimethyl sulfoxide (DMSO; Sigma, less than 0.1%), respectively. After 24 h incubation, the media was removed, cells were washed with 1× PBS, and lysed using passive lysis buffer (Promega). Luciferase activity was detected using the Dual-Luciferase® reporter assay system (Promega) according to the manufacture's instructions and the light output was measured with a luminometer (Analytical Luminescence Laboratory, Monlight® 1500).

2.4. Quantitative real-time PCR

The forward and reverse primers and dual labeled probe were designed by Integrated DNA Technologies (IDT, Coralville, IA) SciTools Primer Quest (Table 2). A homology search using NCBI BLAST was performed to ensure that each primer and/or probe was specific for the target mRNA transcripts/DNA fragments. The probe was labeled with 6-carboxyfluoresceine (FAM) at the 5′ end and Black Hole Quencher-1 (BHQ-1) at the 3′ end and was designed to hybridize to a sequence located between the PCR primers. The primers and dual-labeled probe was synthesized by IDT. To measure GH mRNA levels, total RNA was extracted from GH3 cells using an acid–phenol extraction procedure (Chomczynski and Sacchi, 1987), according to the manufacturer's instructions (Trizol, Invitrogen, Carlsbad, CA). The final RNA pellet was re-suspended in RNase free H_2O and was quantified by UV spectrophotometry. Relative levels of mRNA were determined by quantitative real-time RT-PCR using the Mx3000P™ real time PCR system (Stratagene, La Jolla, CA). The assay for GH expression was performed in 10 μl of 1× QuantiTect Probe RT-PCR Master Mix (Qiagen) containing 400 nM forward primer, 400 nM reverse primer, 200 nM probe, and 1 μg of total RNA. The conditions for cDNA synthesis and target mRNA amplification were performed as follows: one cycle of 50 °C for 30 min; one cycle of 95 °C for 15 min; and 45 cycles each of 94 °C for 15 s, 58 °C for 30 s, and 76 °C for 30 s. All values were normalized to the amplification of β-actin mRNA, which was performed in parallel wells for each treatment. Each treatment was performed in triplicate and real-time RT-PCR analysis was performed in duplicate for each treatment.

To measure DNA immunoprecipitated by the ChIP assay, DNA samples from ChIP were quantified by real-time PCR using the Mx3000P™ real time PCR system (Stratagene) and QuantiTect SYBR Green PCR reagent (Qiagen).

Table 2
Sequence specific primers and probes used for PCR

Purpose	Genes	Accession no.	Primers and probes
Real-time PCR	GH	X12967	F: 5′-AACAAACGATGGTACCCTGCCAGA-3′ R: 5′-CACTGACAGCTTGTG CTGATGGAT-3′
Real-time PCR	CBP	AF410813	F: 5′-TAAT GCAGCCGAGTTAGTGTTGGC-3′ R: 5′-ATCAACAGCTGCAATGGGCATAGG-3′
Real-time PCR	β-Actin	V01217	F: 5′-CCCTCTGAACCCTAAGGCCAACCG-3′ R: 5′-GTGGTGGTGAAGCTGTAGCCACGC-3′
Real-time RT-PCR	GH	X12967	F: 5′-TCCGTGGACAGATCACTGAG-3′ R: 5′-AGCAGGCTGAAGGTCAGG-3′ P: 5′-FAM-TGAGAGTCTGCAGCCATCGCC-BHQ1-3′
Real-time RT-PCR	β-Actin	V01217	F: 5′-TGAACCCTAAGGCCAACCGTGAAA-3′ R: 5′-ATACAGGGACAACACAGCCTGGAT-3′ P: 5′-FAM-ATCATGTTTGAGACCTTCAACACC-BHQ1-3′

RT: reverse transcription; F: forward; R: reverse; P; probe.

The assay was performed in 25 μl of 1× QuantiTect SYBR Green PCR Master Mix (Qiagen) containing 500 nM forward primer, 500 nM reverse primer. The conditions for target DNA amplification were performed as follows: one cycle of 95 °C for 10 min; and 40 cycles each of 94 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s. All values were normalized to the amplification of GH TRE from total input DNA, which was performed in parallel wells for each treatment. Each experiment was performed more than four times. Real-time PCR analysis was performed in duplicate for each treatment.

2.5. Chromatin immunoprecipitation

GH3 cells were prepared and treated as described above. After 24 h of treatments, cells were cross-linked with 1% formaldehyde at room temperature for 10 min. Glycine was added to a final concentration of 125 mM to stop cross-linking. Cells were rinsed twice with cold 1× PBS, collected and incubated for 10 min on ice in cell lysis buffer [5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP-40, 1× Protease Inhibitor Cocktail (Calbiochem Inc., San Diego, CA)] followed by centrifugation to collect nuclei. The nuclei were then resuspended with Nuclei lysis buffer [50 mM Tris–HCl (pH 8.1), 10 mM EDTA, 1% SDS, 1× Protease Inhibitor Cocktail] and incubated 10 min on ice. The resuspended nuclei were then diluted by adding ChIP dilution buffer [16.7 mM Tris–HCl (pH 8.1), 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, 1× Protease Inhibitor Cocktail]. Chromatin was sonicated 10 times for 10 s each (Branson Ultrasonic Corporation, Danbury, CT) followed by centrifugation. Supernatants for TRβ1 and SRC1 immunoprecipitation were pre-cleared with salmon sperm DNA/Protein G agarose (Upstate, New York, NY) and preimmune mouse-IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 40 min at 4 °C. Supernatants for NCoR immunoprecipitation were pre-cleared with salmon sperm DNA/Protein A agarose (Upstate) and preimmune rabbit-IgG (Santa Cruz Biotechnology Inc.) for 40 min at 4 °C. Eighty microliters of supernatant was saved for total input DNA control. Immunoprecipitation was performed overnight at 4 °C with specific antibodies; anti-TRβ1 (sc-738X, Santa Cruz Biotechnology Inc.), anti-NCoR (ab3482, Abcam Inc., Cambridge, MA), and anti-SRC1 (ab84, Abcam Inc.). After immunoprecipitation, 80 μl of salmon sperm DNA/Protein G or A agarose were added and the incubation continued for another 1.5 h. Precipitates were washed sequentially for 5 min each in Low salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 150 mM NaCl], High salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 500 mM NaCl], LiCl immune wash buffer (Upstate). Precipitates were then washed twice with 1× TE (pH. 8.0) and extracted two times with 1% SDS, 0.1 M $NaHCO_3$. Elutes were incubated at 65 °C with 0.25 M NaCl overnight to reverse cross-linking followed by another 1 h incubation at 45 °C with 10 μM EDTA, 40 μM Tris–HCl (pH 6.8) and 2 μg Proteinase K (Sigma). DNA fragments were recovered by phenol/chloroform IAA extraction followed by EtOH precipitation with 20 μg glycogen (Roche, Germany). GH TRE fragments were quantified with real-time PCR.

2.6. Western blot

Follow the procedure for ChIP as described above, and after the final wash of the Protein G agarose beads, we added 2× SDS-PAGE sample buffer (100 mM Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.2% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min. Samples were then subjected to electrophoresis on SDS-polyacrylamide gel followed by transfer to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked 1 h with 5% non-fat dry milk and then incubated with TRβ1 antibody followed by incubation with HRP-conjugated secondary antibody. Bands were visualized by ECL chemiluminescence detection kit (Amersham, Arlington Heights, IL).

2.7. Statistical analysis

Results were analyzed using a one-factor analysis of variance (ANOVA) and Student's *t*-test. Post hoc tests, where appropriate, were performed by Bonferroni's *t*-test where the mean squared error term in the ANOVA table was used as the point estimate of the pooled variance [Graphpad Prism (ANOVA), San Diego, CA].

3. Results

3.1. 4-OH-CB106 binds to TRβ1

To confirm previous reports that 4-OH-CB106 binds to TRs, we defined the binding characteristics of 4-OH-CB106 to human TRβ1. Saturation analysis revealed that T_3 bound to purified hTRβ1 with a K_d of $(0.4399 \pm 0.06512) \times 10^{-9}$ M (Fig. 1A and B). Using the K_d obtained from saturation analysis, a specific K_i was calculated for each of T_3, T_4, Triac, Tetrac, and 4-OH-CB106 (Table 3; Fig. 1C). T_3 and T_4 bound to hTRβ1 with the expected K_i of 1.405×10^{-9} and 2.665×10^{-9}, respectively (Cheek et al., 1999). Additionally, 4-OH-CB106 displaced ^{125}I-T_3 from hTRβ1 with a K_i of 4.706×10^{-5} (Fig. 1C).

3.2. 4-OH-CB106 increases GH mRNA levels in GH3 cells

To test whether 4-OH-CB106 affects GH expression in GH3 cells, we used quantitative real-time RT-PCR to measure GH mRNA levels after treating GH3 cells with 100 nM T_3 or 1×10^{-5} M 4-OH-CB106. We found that GH mRNA levels were

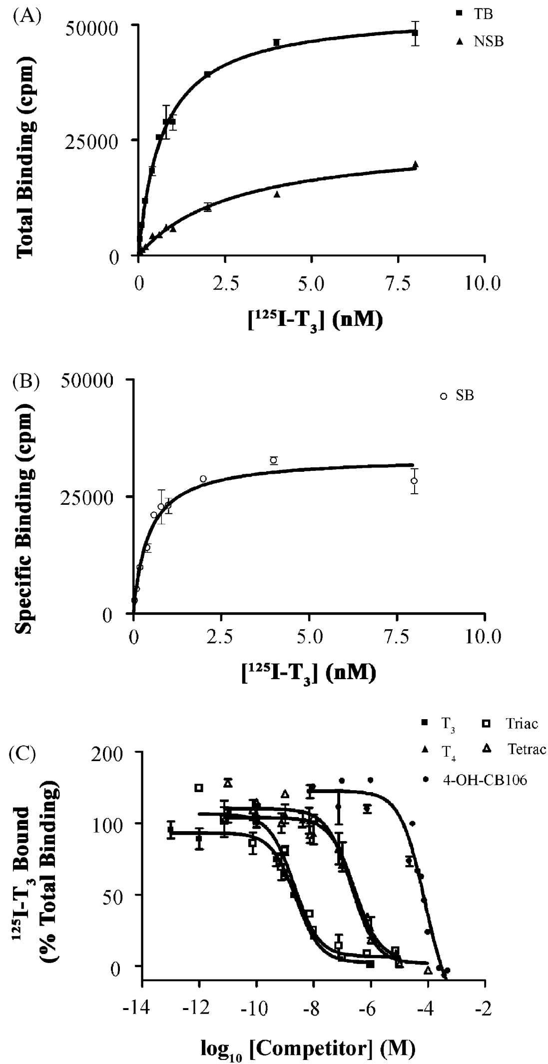

Fig. 1. 4-OH-CB106 binds to hTRβ1. (A) Total binding (TB) obtained by incubating hTRβ1 with increasing concentrations (1.5×10^{-7} to 8×10^{-9} M) ^{125}I-T_3. NSB, non-specific binding. (B) Saturation analysis of ^{125}I-T_3 specific binding (SB) to purified hTRβ1. Bars represent mean ± S.E.M. (C) Competitive binding of known TH agonists and 4-OH-CB106 to purified hTRβ1. Based on saturation analysis, 3×10^{-10} M ^{125}I-T_3 was used in competitive binding experiments with increasing concentrations of T_3, T_4, Triac, Tetrac, or 4-OH-CB106; TR agonists displayed an expected order of binding affinity to hTRβ1 ($T_3 \approx$ Triac > $T_4 \approx$ Tetrac). 4-OH-CB-106 binds to hTRβ1 with 10,000-fold lower affinity. All curves were obtained from results of two experiments and triplicate each. Bars represent mean ± S.E.M.

significantly higher in GH3 cells treated with either T_3 or with 4-OH-CB106, compared to controls (Fig. 2A). T_3 induced a four-fold increase in GH mRNA, whereas, 4-OH-CB106 induced a 1.4-fold increase. To test whether TR mediates these effects on GH expression, cells were transfected with a DR4-tk-Luc or ΔDR4-tk-Luc and subsequently treated with T_3 or 4-OH-CB106. GH3 cells transfected with DR4-tk-Luc exhibited a significant increase in luciferase activity compared to control cells following 100 nM T_3 or 1×10^{-5} M 4-OH-CB106 treatment (Fig. 2B). Interestingly, T_3 induced a ~3.5-fold increase in Luciferase activity, whereas, 4-OH-CB106 induced a ~2.5-fold increase. In contrast, cells transfected with ΔDR4-tk-Luc did not exhibit a significant increase in luciferase activity following T_3 or 4-OH-CB106 treatment (Fig. 2B). To reconcile the quantitative differences in the relative effect of 4-OH-CB106 treatment on GH mRNA levels and on the DR4 alone, we evaluated the response to 4-OH-CB106 on a 250 bp region of the rat GH promoter. Cells transfected with GH250-tk-Luc exhibited a ~1.4-fold increase in luciferase activity (Fig. 2C).

3.3. Chromatin immunoprecipitation (ChIP) assay for GH TRE

We employed chromatin immunoprcipitation (ChIP) to measure TR occupancy of the GH TRE and to determine whether 4-OH-CB106 can affect TR binding to the promoter. In addition, we employed ChIP using antisera against cofactors such as SRC1/NCoR, to determine whether 4-OH-CB106 could alter the relationship between TR and these cofactors.

The TRE on the rat GH promoter is located at −186 to −158 (Crone et al., 1990); therefore, we chose to focus our attention on this region in the ChIP assay (Fig. 3A). To track the degree to which non-specific DNA was isolated in the ChIP assay, we focused on DNA fragments within the cAMP response element binding protein-binding protein (CBP) and β-actin genes. These two genes were selected as negative controls because CBP (10q12) is located on the same chromosome as GH (10q32.1 −q32.2), but in the far distal region, and β-actin (12p11) is located on a different chromosome than GH, and those fragments are not known to contain TR binding sites. Therefore, we did not expect to isolate these DNA fragments following immunoprecipitation (IP) with an antibody against TR.

Accordingly, TRβ1 was successfully immunoprecipitated using anti-TRβ1, as evaluated by Western blot (Fig. 3B). The GH TRE was immunoprecitated with TRβ1, but neither CBP

Table 3
TH receptor agonists and 4-OH-CB106 tested in competitive TR binding assays

Competitor	Abbreviations	IUPAC nomenclature	K_i (M)
Triiodothyronine	T_3	L-3,3′,5-Triiodothyronine	1.405×10^{-9}
Thyroxine	T_4	L-3,3′,5,5′-Tetraiodothyronine	2.665×10^{-7}
Triiodothyroacetic acid	Triac	3,3′,3-Triiodothyroacetic acid	1.234×10^{-9}
Tetraiodothyroacetic acid	Tetrac	3,3′,5,5′-Tetraiodothyroacetic acid	1.248×10^{-7}
4-Hydroxy-PCB106	4-OH-CB106	4′-OH-2,3,3′,4,5-pentaCB	4.706×10^{-5}

CB: chlorinated biphenyl; IUPAC: International Union of Pure and Applied Chemistry.

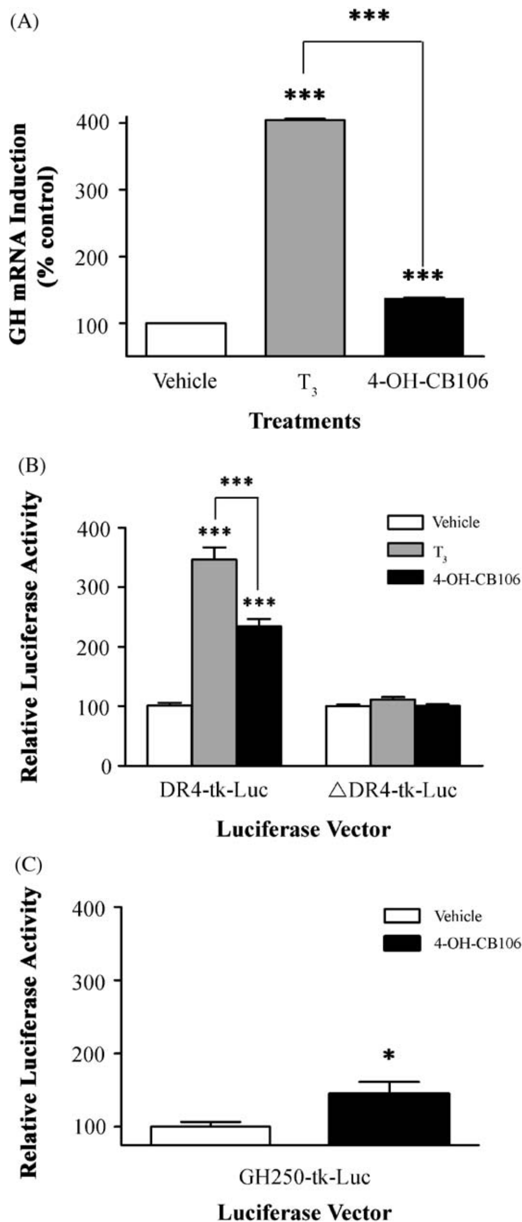

Fig. 2. Effects of 4-OH-CB106 transcriptional regulation by TR. (A) 4-OH-CB106 increases GH mRNA level in GH3 cells. Bars represent mean ± S.E.M. of GH/β-actin mRNA expressed as fold induction over GH3 cells treated with vehicle alone (DMSO). $^{***}p<0.001$, significantly different from control group (vehicle) using ANOVA followed by Bonferroni's multiple comparison test. (B) 4-OH-CB106 increases DR4 TRE luciferase activity but not from the mutated DR4 TRE. Bars represent mean ± S.E.M. of luciferase activity relative to renilla (see text). $^{***}p<0.001$, significantly different from control group (vehicle) using a one-way ANOVA followed by Bonferroni's multiple comparison test. (C) 4-OH-CB106 increases luciferase activity driven by a 250 bp fragment of the native GH TRE. Bars represent mean ± S.E.M. of luciferase activity relative to renilla. $^{*}p<0.05$, significantly different from control group using a Student's t-test. In all cases, treatments were performed in triplicate and final results were obtained from three separate experiments.

nor β-actin was co-precipitated under these conditions (Fig. 3C). Thus, our ChIP assay selectively isolated the GH TRE compared to these negative controls.

Neither T_3 nor 4-OH-CB106 significantly affected the abundance of the GH TRE isolated by ChIP with TRβ1

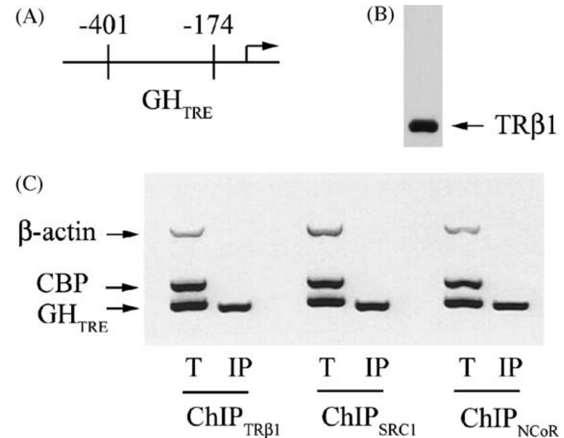

Fig. 3. Validation of chromatin immunoprecipitation (ChIP) assay. (A) The functional TRE on GH promoter (GH_{TRE}) amplified after ChIP. (B) TRβ1 was specifically immunoprecipitated during ChIP procedure and visualized by Western analysis. (C) The GH_{TRE} occupancy by TRβ1, SRC1, or NCoR. Soluble chromatin was prepared from GH3 cells and immunoprecipitated with antibodies against TRβ1 ($ChIP_{TR\beta1}$), SRC1 ($ChIP_{SRC1}$), or NCoR ($ChIP_{NCoR}$). Total input DNA (T) and the final immunoprecipitated DNA (IP) were amplified using pairs of primers that cover the regions of GH_{TRE}, CBP, and β-actin shown in Table 2.

[$F_{3,38}=1.866$; $p=0.1517$] (Fig. 4A). Likewise, neither T_3 nor 4-OH-CB106 influenced the abundance of GH TRE immunoprecipitated with an antibody against SRC1 [$F_{3,23}=2.373$; $p=0.0965$] or NCoR [$F_{3,24}=0.5588$; $p=0.6473$] (Fig. 4B and C).

4. Discussion

The present findings demonstrate that the hydroxylated PCB congener, 4-OH-CB106, can act as a direct agonist on the β1 isoform of the TR to enhance GH expression in GH3 cells. This hydroxylated PCB metabolite bound directly to purified human TRβ1, it increased endogenous GH mRNA in GH3 cells, and it activated TRE-dependent luciferase activity driven by two different TREs. This effect was specific for the TRE inasmuch as 4-OH-CB106 did not activate luciferase activity when driven by a mutant TRE that does not bind the TR. Finally, similar to T_3, this hydroxylated PCB congener did not alter the ability of the TRβ1 to associate with the GH TRE, nor did it affect the association of the co-regulators NCoR and SRC1 with this TRE.

The observation that 4-OH-CB106 increased endogenous GH mRNA in GH3 cells is important because it demonstrates that this PCB metabolite can increase the expression of a direct TH response gene within the context of native chromatin. However, it appears that 4-OH-CB106 interacts with the GH promoter in a manner that is not fully recapitulated by T_3. This interpretation is based on the observation that 4-OH-CB106 exerted a small effect (1.4-fold) on GH mRNA levels compared to T_3 (four-fold), but a greater effect on the DR4 (2.5-fold) compared to T_3 (3.5-fold) in the reporter assay (Fig. 2A and B). We speculate that 4-OH-CB106 exerts a diminished effect on the endogenous GH promoter perhaps because the native promoter contains both a positive and a negative TRE (Kim et al., 1992). Thus, it is possible that 4-OH-CB106 exerts variable effects on transcriptional

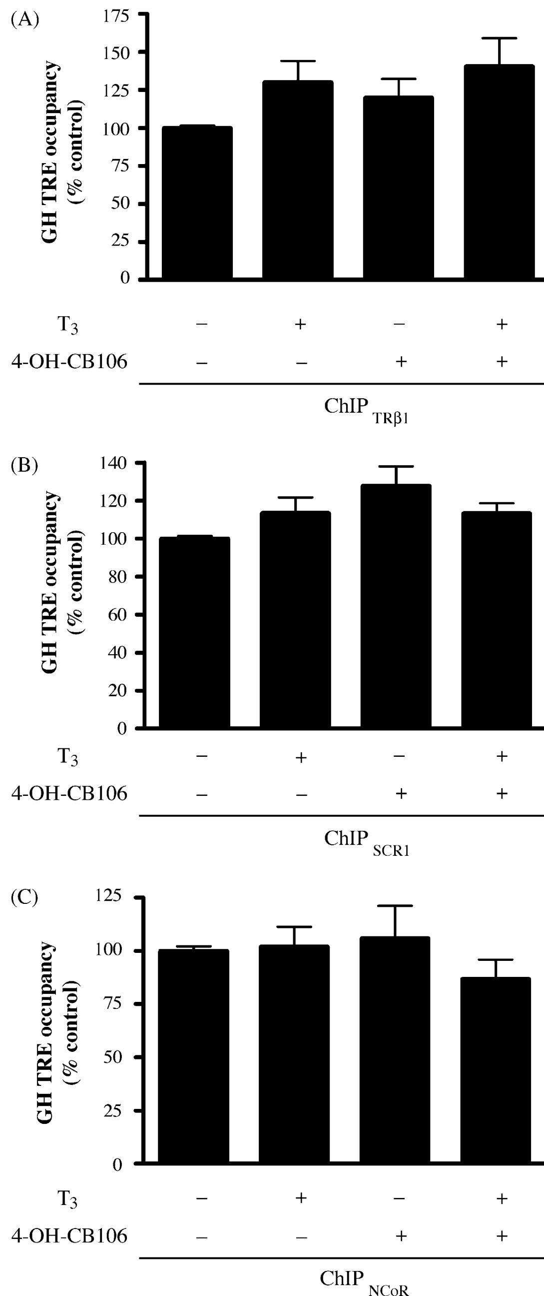

Fig. 4. Neither T_3 nor 4-OH-CB106 affected the TRβ1 interaction with TRE/coregulators in GH3 cells. GH3 cells were treated with 100 nM T_3 and/or 1 ± 10^{-5} M 4-OH-CB106 before harvesting for ChIP assays. Chromatin samples were immunoprecipitated with antibodies against TRβ1 (A), and SRC1 (B) or NCoR (C). Quantification of GH TRE was performed by real-time PCR. Bars represent mean ± S.E.M. of GH TRE occupancy after $ChIP_{TR\beta1}$, $ChIP_{SRC1}$, or $ChIP_{NCoR}$. No statistical significance was determined by ANOVA. In all cases, results were obtained from four experiments in duplicate each.

regulation by the TR depending on the specific enhancer region. Interestingly, 4-OH-CB106 exerted a reduced effect on a 250 bp region of the GH promoter (Fig. 2C), consistent with this concept. A single point mutation in the DR4 TRE present in the ΔDR4 fully abrogated the effect of 4-OH-CB106 on Luciferase activity. Thus, these data indicate that 4-OH-CB106 acts as an agonist on the DR4 TRE, that the presence of additional sequences in the GH 250 promoter may reduce the efficacy of 4-OH-CB106 as an agonist, and that these effects are specific for the TR.

Although these findings are consistent with the interpretation that 4-OH-CB106 is an agonist on the TR, an important potential confounding variable is created by the fact that the TR acts as a constitutive repressor in the unliganded state (Hashimoto et al., 2001; Wondisford, 2003). Thus, if 4-OH-CB106 causes the TR to dissociate from the TRE as observed by Miyazaki et al. (2004) it may be manifested by a small increase in gene expression (i.e., de-repression) without transcriptional activation. This would explain why 4-OH-CB106 exerts a much smaller effect on gene expression than that of T_3. To directly test this hypothesis, we employed the ChIP assay to visualize the effect of 4-OH-CB106 on TR association with the GH TRE, because the quantity of GH TRE immunoprecipitated by an antibody to the TRβ1 reflects the amount of TR associated with this promoter. Our optimized $ChIP_{TR\beta1}$ assay selectively immunoprecipitated the GH TRE; however, neither T_3 nor 4-OH-CB106 affected TR binding to the GH promoter. Thus, these findings support the interpretation that 4-OH-CB106 acts as a direct TR agonist, but do not support the observation of Miyazaki et al. (2004) that 4-OH-CB106 causes the partial dissociation of the TR from the TRE. However, Miyazaki et al. used electrophoretic mobility shift assay (EMSA) to visualize the ability of 4-OH-CB106 to cause a partial dissociation of the TR from the DR4 TRE. It is theoretically possible that 4-OH-CB106 exerts a similar action on the TR in native chromatin but that the TR remains in close proximity to the DNA such that it is cross-linked to associated DNA and immunoprecipitated in the ChIP assay. Liu et al. (2006) have shown that the TR and SRC1 appear to "cycle" on and off the GH TRE in GH3 cells in a relatively short period (min), suggesting that the TR remains in close proximity to the TRE. Thus, it remains to be determined whether PCBs can affect the temporal pattern of receptor/cofactor interaction with the TRE or if this temporal pattern is important for physiological control.

Surprisingly, neither T_3 nor 4-OH-CB106 affected our ability to immunoprecipitate the GH TRE using antibodies specific for NCoR or SRC1. This observation was surprising because a great deal of work indicates that T_3 binding to the TR causes a conformational change in the "hinge" region of the receptor that reduces the ability of the receptor to bind co-repressors such as NCoR and to "recruit" co-activators such as SRC1 (Aranda and Pascual, 2001). Thus, if NCoR and SRC1 are physically "released" and "recruited" in native chromatin as they appear to be in cell-free or transient-transfection systems, then we expected to observe a simultaneous decrease in NCoR association with the TR and an increase in SRC1 association with the TR on the GH TRE in the presence of T_3. Our finding that $ChIP_{NCoR}$ and $ChIP_{SRC1}$ were not associated with predicted changes in the abundance of the GH TRE following immunoprecipitation is not likely to be a technical problem with the assay because the two negative controls used in the current experiment – CBP and β-actin – were not immunoprecipitated with antibodies to TRβ1, NCoR or SRC1.

In contrast, there are two possible explanations for these findings. First, it is possible that NCoR and SRC1 are released from the TR in the liganded and unliganded state, respectively, but

remain within the transcriptional complex containing TRβ1 in association with the GH TRE. Thus, following cross-linking, the NCoR and SRC1 epitopes remain available to immunoprecipitate the GH promoter. If so, ChIP will not be capable of studying the relationship between the TR and these co-factors in native chromatin. In support of this interpretation, Li et al. (2002) report that NCoR can bind directly to the co-activator ACTR, and that the molecular site at which they interact is different from the site responsible for nuclear receptor binding. Also they reported that NCoR facilitates an interaction between unliganded TRβ and ACTR, suggesting that ligand occupancy to the receptor is not a determinant of coactivator/corepressor selection. A second possibility is that these cofactors, which interact with other nuclear receptors at other sites along this GH promoter, are unaffected by T_3 and/or PCB treatment and thus remain available for the IP step. If so, co-factors bound to other nuclear receptors will confound our ability to observe the effect of T_3 and/or PCB on cofactor recruitment to the TR itself. Although plausible, this would require close proximity to the TR because the sonication step disrupts the DNA into approximately 800 bp fragments. These issues will require further study to resolve.

It is important to be cautious when considering the potential physiological significance of our present findings. On one hand, the finding that 4-OH-CB106 is a direct agonist on the TR is consistent with the observation that PCB exposure *in vivo* can exert TH-like effects (i.e., agonistic) in the developing rodent brain (Gauger et al., 2004; Zoeller et al., 2000) and may indicate that PCBs exert similar actions in humans. In addition, because 4-OH-CB106 can also bind to serum thyroxine binding proteins, such as transthyretin (TTR) (Chauhan et al., 2000; Cheek et al., 1999; Lans et al., 1993; Lans et al., 1994; Malmberg et al., 2004) our findings are consistent with the proposal of some authors that binding to TTR may be a predictor of binding to other thyroid hormone-binding proteins (Marchesini et al., 2006).

On the other hand, the low affinity of 4-OH-CB106 to the TRβ1 is difficult to reconcile with agonistic effects at concentrations found in humans (Takser et al., 2005). For example, a recent study by Lackmann et al. (2004) found 6-week old breast-fed had serum PCB levels of 1.19 μg/L, which was significantly higher than the 0.29 μg/L found in bottle-fed infant serum. Furthermore, Kalantzi et al. (2004) reported that total PCB levels in breast milk in the U.K. ranged from 26 to 530 ng/g lipid, translating to a daily infant intake of 6.24–2067 ng/kg. Thus, blood levels of PCBs in humans do not appear to be high enough for any single congener to exert agonistic effects on the TR.

Despite the low affinity of 4-OH-CB106 for the TR, there are several reasons to entertain the possibility that individual hydroxylated PCBs may exert actions on the TR at environmental concentrations. First, we focused on 4-OH-CB106 because several other groups have used this congener in their studies and, therefore, we could compare our findings to previous reports. However, this PCB congener is not routinely found in human samples; rather, 4-OH-CB107 has been identified in human and wildlife samples as a common metabolite of CB118 and CB105 (Sjodin et al., 2000) that is preferentially found in fetal plasma and brain (Morse et al., 1996). Thus, it is possible that other PCB metabolites exhibit a higher affinity for the TR and that they are found in cells and tissues at concentrations that are higher than predicted based on serum concentrations (De Saeger et al., 2005; Sugiyama et al., 1995; Falandysz et al., 1994; Chu et al., 2003).

In addition, the transcriptional potency of these TR analogues may not be fully predicted by their low affinity for the receptor (Chen et al., 2000; Gee et al., 1999). Weyts et al. (2004) have described modifications to a Vitamin D receptor (VDR) ligand that do not alter affinity for the VDR but enhance VDR-mediated gene transcription by as much as 50-fold. Although the transcriptional potency of 4-OH-CB106 on GH expression or DR4 promoter activity in the present study is consistent with its observed affinity for the TRβ1, this may not be the case for all TREs. In addition, transcriptional potency for a nuclear receptor ligand depends on the relative abundance of receptor, coregulators and target genes (Nettles and Greene, 2005). Thus, 4-OH-CB106, in combination with or in addition to other PCBs in complex mixtures to which humans are exposed, may be potent enough to exert TH-like effects on TR-regulated gene expression at environmental levels.

In conclusion, the present study demonstrates that a single hydroxylated PCB congener, 4-OH-CB106, can act as a direct TR agonist on the rat TRβ1 in regulating GH gene expression. If this PCB congener, or if other PCB congeners, can act as inappropriate TR agonists during brain development, one would predict adverse outcomes. For example, TH regulates the balance of production of oligodendrocytes and astrocytes in areas of white matter (Sharlin et al., 2006). Thus, inappropriate TH signaling by PCBs could produce an abnormal balance of these two cell types. TH administration as well as PCB exposure to pregnant rats increases the abundance of HES1 mRNA in the fetal cortex on gestational day 16 (G16) (Bansal et al., 2005), which plays an important role in fate specification of cortical neurons. Hydroxylated PCBs may interact directly with TRs to exert this effect. However, and perhaps more importantly, the possibility that PCBs may exert different effects on TR actions on different TH-regulated genes, suggests that PCBs may produce a mosaic of effects on TH signaling in different tissues or at different times during development. This likely possibility will be important to identify and define.

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