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Pituitary specific retinoid-X receptor ligand interactions with thyroid hormone receptor signaling revealed by high throughput reporter and endogenous gene responses

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A B S T R A C T

Disruption of thyroid hormone (TH) signaling can compromise vital processes both during development and in the adult. We previously reported on high-throughput screening experiments for man-made TH disruptors using a stably integrated line of rat pituitary cells, GH3.TRE-Luc, in which a thyroid hormone receptor (TR) response element drives luciferase (Luc) expression. In these experiments, several retinoid/rexinoid compounds activated the reporter. Here we show that all-trans and 13-cis retinoic acid appear to function through the heterodimer partners of TRs, retinoid-X receptors (RXRs), as RXR antagonists abrogated retinoid-induced activation. The retinoids also induced known endogenous TR target genes, showing good correlation with Luc activity. Synthetic RXR-specific agonists significantly activated all tested TR target genes, but interestingly, retinoid/rexinoid activation was more consistent between genes than the extent of T3-induced activation. In contrast, the retinoids neither activated the Luc reporter construct in transient transfection assays in the human hepatocarcinoma cell line HuH7, nor two of the same T3-induced genes examined in pituitary cells. These data demonstrate the suitability and sensitivity of GH3.TRE-Luc cells for screening chemical compound libraries for TH disruption and suggest that the extent of disruption can vary on a cell type and gene-specific bases, including an underappreciated contribution by RXRs.

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1. Introduction

Thyroid hormone (TH) signaling regulates critical aspects of development and adult physiology (Pascual and Aranda, 2013). For example, during mammalian development, TH controls maturation of the cerebellum (Fauquier et al., 2014), cochlea and hearing (Rusch et al., 2001), and opsin expression for color vision in retinal cone cells (Ng et al., 2001). In the adult TH is responsible for controlling the basal metabolic rate, renal clearance, cardiac function, including heart rate, and fertility (Unuane et al., 2011; Galli et al., 2010; McAninch and Bianco, 2014). TH is also necessary to trigger metamorphosis in most amphibians and in flatfish (Furlow and Neff, 2006; Sachs et al., 2000; Schreiber, 2013). Therefore endocrine disruption of TH signaling has the potential to be a major concern for both human health and environmental stability.

TH signaling is primarily mediated through the thyroid hormone receptors (TRs), which are members of the nuclear receptor superfamily of transcription factors. TRs bind to specific DNA sequences termed thyroid hormone receptor response elements (TREs) both in the absence and presence of TH (reviewed in Cheng et al. (2010), Zhang and Lazar (2000)). The unliganded apo-receptor may either repress or activate transcription in a gene specific manner, but the liganded TR will then do the opposite. The molecular mechanisms by which unliganded TR represses transcription and TH-bound TR activates transcription are the best understood (Cheng et al., 2010). In this scenario, chemical compounds with agonist activity will cause gene activation, and antagonists will either prevent activation and/or increase repression. Vertebrates have two different genes for TRs, TRα and TRβ, and birds and mammals express at least two hormone-binding isoforms of TRβ through alternative mRNA splicing and promoter usage (Flamant and Gauthier, 2013).

Proper TH signaling requires a euthyroid state, with both hypothyroidism and hyperthyroidism having severe health consequences. Therefore, thyroid hormone synthesis by the thyroid gland is very closely controlled through a negative feedback loop of the hypothalamus–pituitary–thyroid axis. This axis makes

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pituitary cells particularly sensitive to TH, and therefore a good cell type choice for screening chemical compound libraries for TH disruption. GH3 is an epithelial-like pituitary cell line from a female rat pituitary tumor that is well characterized in its endogenous expression of all hormone-binding TRs (TRα1, TRβ1 and TRβ2) and response to TH (Lazar and Chin, 1988). Previously, we made a stable, clonal cell line of GH3 cells, GH3.TRE-Luc, which have an integrated canonical TRE element (2X-taDR4) driving a destabilized luciferase reporter gene (Freitas et al., 2011). Destabilization of reporter proteins involves the fusion of degradation signals to the luciferase gene; the faster degradation allows for a faster and higher magnitude response to rapid transcriptional changes (Li et al., 1998). TH induces Luc activity greater than 10-fold at sub-nanomolar concentrations in a dose dependent manner (Freitas et al., 2011).

Quantitative high-throughput screening (qHTS) of a miniaturized (1536-well format) version of the TRE-driven luciferase (Luc) assay that tested the 1280 compounds of the LOPAC library (Library of Pharmacologically Active Compounds) and 1408 chemicals from the National Toxicology Program collection (NTP) for TH disruption (Freitas et al., 2014) revealed that the synthetic RXR agonist bexarotene and the natural retinoids all-trans retinoic acid (ATRA) and 13-cis retinoic acid (13-cis RA) were agonists (Freitas et al., 2014). ATRA is a ligand for the retinoic acid receptors (RARs), and 13-cis RA is often considered a pro-drug for RAR signaling, as treatment with 13-cis RA causes activation of RAR target genes, and 13-cis RA can be converted to ATRA intracellularly (Lanvers et al., 1998; Armstrong et al., 2005a,b). However, the synthetic pan-RAR agonist TTNPB, did not induce Luc activity (Freitas et al., 2014), suggesting that the ATRA and 13-cis RA were not acting through RARs to activate the Luc gene. TRs form heterodimers with RXRs, which increases their affinity for DNA binding, especially in the presence of TH (Zhang and Kahl, 1993). However, an RXR agonist is generally not thought to affect the transcriptional outcome either in the absence or presence of TH, a situation referred to as non-permissive heterodimerization (Forman et al., 1995). RXR forms heterodimers with many members of the nuclear receptor family. Some of the resulting heterodimers are permissive, meaning that an RXR agonist can activate the heterodimer even in the absence of its partner's ligand, and then when both ligands are present, a synergistic response is seen (Shulman et al., 2004). Other researchers have found evidence for a partial permissiveness in the TR–RXR heterodimer using transient transfection reporter experiments, which was especially prevalent in pituitary cell lines and choriocarcinoma cells (Castillo et al., 2004; Li et al., 2002).

For this report, we examine whether these qHTS positive hit retinoids were simply activating the idealized TRE driven reporter gene, or could also activate a range of endogenous TR target genes in GH3.TRE-Luc cells. Further, we examine whether that activation is mediated through RXRs functioning in a permissive role in these cells.

2. Materials and methods

2.1. Materials

T3, T0901317 and bexarotene were purchased from Sigma Aldrich (St. Louis, MO); ATRA, 13-cis RA, 9-cis RA and TTNPB were purchased from Tocris Bioscience (Bristol, UK); LG100268 and LG100208 were kindly provided by M. Leibowitz (Ligand Pharmaceuticals, San Diego). DMEM/F12 and DMEM were from Hyclone (Thermo Scientific, Waltham, MA) and Gibco FBS was from Life Technologies (Thermo Scientific).

2.2. Cell culture

GH3 and GH3.TRE-Luc (Freitas et al., 2011) were grown in DMEM-F12/10% FBS and HuH7 cells in DMEM/10% FBS in a 5% CO_2 humidified incubator at 37 °C. GH3 and GH3.TRE-Luc cells were passaged every 5 days at 1/5 dilution, and HuH7 cells were passaged every 2–3 days at 1/5 dilution.

2.3. Luciferase assays

GH3.TRE-Luc cells were plated in 24-well plates at 1.5×10^5 cells/well in growth medium. After 24 h growth, cells were washed with PBS and 0.5 ml PCM, a serum-free medium preparation (Sirbasku et al., 1991) was applied. After 24 h incubation in PCM, the PCM was replaced with PCM + ligands or vehicle (DMSO) in duplicate. DMSO concentration was kept less than 0.1%. After 24 h treatment with ligand, cells were washed with PBS and lysed with 100 μl of 1X Reporter Lysis Buffer (Promega, Madison, WI) and frozen at −80 °C to aid lysis. Lysates were thawed at room temperature with mild rotation on a platform rotator and luciferase activity was measured using 5 μl of lysate and 20 μl of Luciferase Assay Reagent (Promega) in a Chameleon V microplate reader (Hidex, Turku, Finland). Lysate protein concentrations were measured for normalization using the BCA Assay Kit from Thermo Fisher Scientific (Rockford, IL). Relative luciferase units were calculated by dividing raw luciferase units by the protein concentration. Each duplicate assay was repeated at least three times independently.

2.4. Transient reporter assays

GH3 cells were plated in 24-well plates at 2.5×10^5 cells/well in growth medium. After 24 h, cells were washed with PBS and 0.4 ml PCM was applied. HuH7 cells were treated similarly except 3×10^4 cells/well were plated and DMEM/10% hormone-stripped FBS was used in lieu of PCM at all steps. The cells were transfected with 100 ng of pGL4-SV40-2XDR4-Luc or pGL4-SV40-Luc (null), 50 ng of pCMV-LacZ as an internal transfection control and 350 ng of pUC18 as DNA filler, using 0.75 μl of Lipofectamine 3000 and 1 μl of P3000 Reagent per transfection (Life Technologies, Grand Island, NY) following the manufacturer's protocol. After 24 h, the medium was replaced with PCM + ligands or vehicle (DMSO) and incubated for an additional 24 h to induce luciferase activity. Cells were lysed and luciferase was assayed as above. β-galactosidase activity was quantified as described (Mengeling et al., 2005). Relative luciferase values were calculated by dividing raw luciferase units by β-galactosidase units. Each transfection was performed independently at least 3 times.

2.5. Gene expression assays

GH3.TRE-Luc cells were plated in 6-well plates at 8.0×10^5 cells/well in growth medium. After 24 h growth, cells were washed with PBS and 2.0 ml PCM was applied. After 24 h incubation in PCM, the PCM was replaced with PCM + ligands or vehicle (DMSO) for 24 h. HuH7 cells were treated similarly except 1×10^5 cells/well were plated and DMEM/10% hormone-stripped FBS was used in lieu of PCM. All cells were harvested in 1 ml Qiazol (Qiagen, Valencia, CA) and stored at −80 °C for less than 1 week. Total RNA was extracted following the manufacturer's protocol and quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE). cDNA was synthesized from 1 μg of total RNA using either the Quantitect Reverse Transcription Kit (Qiagen) or the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Life Technologies) and following the manufacturer's instructions; the kits gave identical results. qPCR was performed

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