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Research paper

Ectopic cross-talk between thyroid and retinoic acid signaling: A possible etiology for spinal neural tube defects

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ABSTRACT

Previous studies have highlighted the connections between neural tube defects (NTDs) and both thyroid hormones (TH) and vitamin A. However, whether the two hormonal signaling pathways interact in NTDs has remained unclear. We measured the expression levels of TH signaling genes in human fetuses with spinal NTDs associated with maternal hyperthyroidism as well as levels of retinoic acid (RA) signaling genes in mouse fetuses exposed to an overdose of RA using NanoString or real-time PCR on spinal cord tissues. Interactions between the two signaling pathways were detected by ChIP assays. The data revealed attenuated DIO2/DIO3 switching in fetuses with NTDs born to hyperthyroid mothers. The promoters of the RA signaling genes *CRABP1* and *RARB* were ectopically occupied by increased RXRG and RXRB but displayed decreased levels of inhibitory histone modifications, suggesting that elevated TH signaling abnormally stimulates RA signaling genes. Conversely, in the mouse model, the observed decrease in *Dio3* expression could be explained by increased levels of inhibitory histone modifications in the *Dio3* promoter region, suggesting that overactive RA signaling may ectopically derepress TH signaling. This study thus raises in vivo a possible abnormal cross-promotion between two different hormonal signals through their common RXRs and the subsequent recruitment of histone modifications, prompting further investigation into their involvement in the etiology of spinal NTDs.

1. Introduction

Thyroid hormones (THs), including T3 and T4, are vital for neural development (Flamant and Samarut, 1998; Remaud et al., 2014); dysfunction of maternal THs during pregnancy is linked to a form of neural tube defects (NTDs) (Oakley et al., 1998; Reddy et al., 2010; Monroy-Santoyo et al., 2012). NTDs constitute a class of common and severe anomalies affecting 0.5–2 out of 1000 pregnancies worldwide, with clinical manifestations including both spinal and cranial phenotypes.

3, 3, 5-triiodo-L-thyronine (T3) is the active TH ligand and its production is a critical step in TH-driven gene induction (Davey et al., 2008; Nakane and Yoshimura, 2014). Thyroxine (T4) is converted to T3 by the enzyme 5-deiodinase (DIO2), while DIO3 degrades T3 to the inactive diiodo-L-thyronine form (T2) and converts T4 to reverse T3 (rT3) (Hernandez et al., 2012). Thyroid-stimulating hormone (TSH) can affect normal TH synthesis and secretion in response to feedback from circulating THs. Therefore, T3 concentrations are determined by the balance between TSH and DIO2/DIO3 switching. The cellular effects of THs are classically mediated by thyroid receptor (TR) isoforms, which belong to the nuclear hormone receptor superfamily and are encoded by the *THRA* and *THRB* genes. T4 and T3 reach TRs through the plasma membrane with the aid of the selective TH transporter SLC16A2 (Kogai et al., 2010; Schweizer et al., 2014). Follow ligand binding, TRs heterodimerize with retinoid X receptors (RXRs), of which there are three subtypes, RXRA, RXRB and RXRG. Moreover, RXRs can also partner with another type of specific nuclear hormone receptor, the retinoic acid (RA) receptor (RAR), which is activated by RA (Persaud et al., 2013).

RA is essential for controlling the patterning and differentiation of neural development. Application of an overdose of RA to pregnant mice on day 8 of gestation caused the fetuses to develop a spina bifida phenotype (Alles and Sulik, 1990). In the nucleus, RA activates RAR, which forms heterodimers with RXRs. Similar to the TR/RXR heterodimers, these heterodimers act as transcription factors and bind to hormone-response elements (HREs) to further recruit histone-modifying enzymes and regulate chromatin structure (Kolarcik and

Abbreviations: NTD, neural tube defect; TH, thyroid hormones; RA, retinoic acid; T3, 3, 3, 5-triiodo-L-thyronine; T2, diiodo-L-thyronine; rT3, reverse T3; TR, thyroid receptor; RXRs, retinoid X receptors; RAR, retinoic acid receptor; HRE, hormone-response elements; ER, everted repeats; DR, direct repeats; IR, inverted repeats; ChIP, chromatin immunoprecipitation; fT3, free T3; fT4, free T4; SHH, sonic hedgehog.

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Bowser, 2012; Bohnsack and Kahana, 2013). HREs contain a special sequence structure composed of everted repeats (ERs), direct repeats (DRs) or inverted repeats (IRs) (Helsen and Claessens, 2014).

Cross-talk between TH and RA signaling has been documented under physiological conditions. Kogai et al. proposed that all-trans-RA or RAR promotes the uptake of THs via transcriptional upregulation of SLC16A2 during extraembryonic endoderm development and neural differentiation (Kogai et al., 2010). RA can rescue the neural crest migration defects induced by decreased TH signaling and partially mediated by RXRs (Bohnsack and Kahana, 2013). These observations suggest that RA may play a positive role in activating TH signaling. However, in mouse embryonic stem cells, unliganded Thra has an inhibitory effect on the RA response, inhibiting Rarb expression and modulating RA-stimulated neural differentiation (Lee et al., 1994). Thus, it is important to determine whether and how these two hormonal pathways dysregulate each other in the context of NTDs.

The present study shows that in human spinal NTDs, TH signaling may be influenced by maternal hormone levels, and that the inhibitory histone modifications recruited by RXRs show abnormally low enrichment on RA signaling genes. Conversely, in murine NTDs induced by an overdose of RA, H3K4ac was ectopically enriched on TH signaling genes. These findings highlight the interplay between two signaling pathways in the presence of excessive stimuli.

2. Materials and methods

2.1. Human subjects

Stillborn NTD cases from Shanxi Province in northern China in 2009 were used as subjects. The surgical details were described previously (Zhang et al., 2013). Spinal cord tissues from three subjects with spinal NTDs and four ethnicity- and age-matched controls were analyzed. The study was conducted in accordance with the Declaration of Helsinki. The Committee on Medical Ethics of the Capital Institute of Pediatrics (Beijing, China) approved this study (Permit no: SHERLLM2014002). Written informed consent was obtained from the parents on behalf of the fetuses.

2.2. Animals

A model of fetal spina bifida aperta was established by intraperitoneal injection of pregnant mice with all-trans RA injection at E8. Spinal cord tissues were collected from twenty-one fetal mice with spina bifida aperta phenotypes and 10 normal fetuses. All experiments on animals were conducted in accordance with the standards of the Capital Institute of Pediatrics Ethics Committee (Permit no: SYXK 2008–0011).

2.3. RNA extraction and mRNA detection

Total RNA was extracted from the spinal cord tissues of humans or mice using the RNeasy Mini kit according to the manufacturer's instructions (Cat. no.74104, Qiagen). Real-time qPCR assays were performed to determine the mRNA levels of target genes in mice. The *Gapdh* gene was used as a reference for each sample. For the details of the primers please see in Table S1.

The NanoString nCounter detection method was used to examine transcript levels in human tissues. Hybridizations were performed according to the NanoString Gene Expression Assay manual. Approximately 100 ng of each RNA sample was mixed with 20 μL of nCounter Reporter probes (Table S2) and 5 μL of nCounter Capture probes in hybridization buffer. The purified target/probe complexes were eluted and immobilized in a cartridge for data collection, which was conducted in the nCounter Digital Analyzer. The results were normalized to the *GAPDH*, *CLTC* and *GUSB* genes.

2.4. Measurement of serum TH levels

Maternal serum levels of T3, T4 and TSH were assayed with the Hormone Chemiluminescence Quantitative Immunoassay Kit (BOSON Biotech. CO., Xiamen, China) and read out by VARIOSKAN FLASH (Thermo Scientific, USA). The normal reference ranges were 1.45–3.48 pg/mL for fT3, 0.71–1.85 ng/dL for fT4 and 0.49–4.67 mIU/L for TSH.

2.5. Chromatin immunoprecipitation (ChIP) assay

The MAGnify™ ChIP system was purchased from Invitrogen (USA). Briefly, chromatin products were incubated with 4 μg of anti-RXRA (3085, Cell Signaling Technology (CST), USA), anti-RXRB (8715, CST), anti-RXRG (5629, CST), anti-H3K4ac (ab113672, Abcam, Cambridge, UK), anti-H3K4me (ab106165, Abcam), anti-H3K9me3 (ab8898, Abcam) or anti-H3K27me3 (17–622, Millipore, Billerica, USA) antibodies. The immunoprecipitated DNA was analyzed using real-time PCR. The primer pairs were designed based on HRE sequences within the target gene promoters (Tables S3 and S4). A mouse or rabbit IgG antibody was used as a negative control.

2.6. Statistical analysis

All experiments were performed at least in triplicate. The results are presented as the means ± S.E. Statistical analyses were conducted using Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Maternal serum hyperthyroidism is associated with abnormal active TH signaling

Three human fetuses subjects affected with spinal NTDs were described in Table 1. We assayed maternal serum levels of free T3 (fT3), free T4 (fT4) and TSH via a Chemiluminescence Quantitative Immunoassay. The assays revealed high maternal fT3 levels or low maternal fT4 or TSH levels at the gestational week indicated in Table 1, indicating a tendency toward hyperthyroidism. In cases, the attenuated DIO2/DIO3 switching was observed, along with increased expression of *THRB* but slightly decreased levels of *THRA* (Fig. 1). These results suggest that cases of human spinal NTDs associated with maternal serum hyperthyroidism exhibit present elevated TH signaling and T3 degradation.

3.2. Ectopic repressive histone modifications in the promoters of RA signaling genes

Since TR/RXR heterodimers can recruit histone-modifying enzymes to regulate transcription (Bernal, 2007), to elucidate the possible interplay between TH signaling and RA signaling in the presence of abnormal stimuli in spinal NTDs, we detected the enrichment of RXRs and four histone modifications in the promoters of *CRABP1* (which transports RA into the nucleus) and *RARB* (one component of the RA receptor). In a DR sequence (5′-GGATCACGAGGTCA-3′) in the *CRABP1* promoter, increased enrichment of RXRG was accompanied by downregulated H3K4ac and subsequent H3K9me3 occupancy (Xhemalce and Kouzarides, 2010) (Fig. 2A). In the *RARB* promoter, a significant increase in RXRB enrichment was observed in one DR sequence (5′-GGTTCACC GAAAGTTCA-3′) (Fig. 2B), but not the other (5′-GGGTCATTTGAAGG TTA-3′) (Fig. 2C), this change was accompanied by decreased enrichment of the repressive H3K4ac and H3K27me3 (Fig. 2B). Furthermore, our NanoString results indicated that the mRNA levels of *CRABP1* and *RARB* were upregulated by 1.93- and 2.21-fold, respectively (Fig. 2D). We propose that in human spinal NTDs, excessive TH signaling activity interacts with RA signaling genes and may abnormally prompt the derepression of RA signaling.