Circadian Regulation of Tshb Gene Expression by Rev-Erbα (NR1D1) and Nuclear Corepressor 1 (NCOR1)*

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Background: Basal metabolic rate is regulated by thyroid hormone; the mechanism is unknown.

Results: NCOR1 and Rev-Erbα enrich at different sites from thyroid hormone receptor on the Tshb promoter.

Conclusion: NCOR1 and Rev-Erbα interact to regulate circadian expression of Tshb mRNA independent of thyroid hormone.

Significance: This novel role of Rev-Erbα in Tshb expression reveals new links between circadian rhythms and metabolism.

Thyroid hormones (THs) are critical for development, growth, and metabolism. Circulating TH levels are tightly regulated by thyroid-stimulating hormone (TSH) secretion within the hypothalamic-pituitary-thyroid axis. Although circadian TSH secretion has been well documented, the mechanism of this observation remains unclear. Recently, the nuclear corepressor, NCOR1, has been postulated to regulate TSH expression, presumably by interacting with thyroid hormone receptors (THR) bound to TSH subunit genes. We report herein the first in vitro study of NCOR1 regulation of TSH in a physiologically relevant cell system, the TαT1.1 mouse thyrotroph cell line. Knockdown of NCOR1 by shRNA adenovirus increased baseline Tshb mRNA levels compared with scrambled control, but surprisingly had no affect on the T3-mediated repression of this gene. Using ChIP, we show that NCOR1 enriches on the Tshb promoter at sites different from THR previously identified by our group. Furthermore, NCOR1 enrichment on Tshb is unaffected by T3 treatment. Given that NCOR1 does not target THR on Tshb, we hypothesized that NCOR1 targeted Rev-Erbα (NR1D1), an orphan nuclear receptor that is a potent repressor of gene transcription and regulator of metabolism and circadian rhythms. Using a serum shock technique, we nuclearencorrelated TαT1.1 cells to study circadian gene expression. Post-synchronization, Tshb and Nr1d1 mRNA levels displayed oscillations that inversely correlated with each other. Furthermore, NR1D1 was enriched at the same locus as NCOR1 on Tshb. Therefore, we propose a model for Tshb regulation whereby NR1D1 and NCOR1 interact to regulate circadian expression of Tshb independent of TH negative regulation.

Thyroid hormones (THs), T4 and T3, play an integral role in development, growth, and cellular metabolism (1–3), and circulating levels of THs are maintained within a narrow range by a finely tuned negative feedback system involving the hypothalamic-pituitary-thyroid (HPT) axis (4). Thyrotropin-releasing hormone (TRH) secreted from the hypothalamus stimulates pituitary thyrotrhops to produce biologically active thyroid stimulating hormone (TSH), which stimulates the thyroid gland to synthesize and secrete THs. Importantly, T3 exerts negative feedback at both the level of the pituitary and hypothalamus, thus completing the feedback loop (5). T3 action is classically thought to be mediated via THRs (6), which are members of the nuclear receptor superfamily and bind DNA both in the absence and presence of T3 (7–9). On positively regulated genes, the THR is thought to interact with corepressors, principally NCOR1, which is released and replaced by coactivators after T3 binding (10, 11). In contrast, less is known about mechanisms of genes repressed by T3. We are particularly interested in establishing mechanisms by which Tshb expression is regulated.

Circadian rhythms are fundamental phenomena in most living organisms whereby behavior and biological function are regulated through an autonomous clock. Control of this rhythm has been traced to a central clock in the suprachiasmatic nucleus of the hypothalamus (12). Disruptions of clock mechanisms are thought to be important in disorders of sleep, metabolism, and even cancer (13–15). The circadian cycle is triggered by a CLOCK/BMAL1 heterodimer that is regulated under a negative feedback loop mediated by the orphan nuclear receptor Rev-Erbα (NR1D1) (16–18). Feng et al. (19) mapped a NR1D1 cistrome in mouse liver, showing thousands of binding sites that have a rhythm, which correlate to the oscillating expression of NR1D1.

TSH and T3 secretion are also known to follow a photoperiodic circadian rhythm, with a nadir during the day and a peak secretory activity just before sleep (20). Interestingly, TSH may

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3 The abbreviations used are: TH, thyroid hormone; T3, 3,3',5-triiodo-L-thyronine; T4, thyroxine; NCOR1, nuclear corepressor 1; TSH, thyroid stimulating hormone; THR, TH receptor; TRH, thyrotropin-releasing hormone; qPCR, quantitative PCR.
induce the expression of type 2 iodothyronine deiodinase in the hypothalamus, which enzymatically converts the prohormone T₄ into its bioactive T₃ product, providing an additional mechanism for controlling the HPT axis (21, 22). Given that this diurnal rhythm of TSH is disrupted in states as diverse as depression, poorly controlled diabetes, and mostly importantly, after pharmacologic T₄ replacement to hypothyroid patients, further elucidation of this mechanism is warranted (23, 24).

Despite characterization of TSH diurnal rhythm in physiologic and pathologic states over many years, the upstream regulators are yet to be well characterized. We have established an appropriate and physiologically relevant mouse cell line model to study regulation of appropriate and physiologically relevant mouse cell line model. Further characterization of TSH diurnal rhythm in physiologic and pathologic states over many years, the upstream reg-

EXPERIMENTAL PROCEDURES

Cell Culture and Hormone Treatments—TaT1.1 cells were plated in DMEM (Corning Cellgro, Manassas, VA) containing 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Before the cells were seeded, the plates were coated with Matrigel (BD Biosciences) to facilitate adhesion. Matrigel was diluted 30-fold with Dulbecco’s PBS (Invitrogen) before coating the plates. Cells were maintained at 37 °C in an environment of 5% CO₂. Treatment of cells with either T₃, TRH, or SR9011 (Sigma) was performed for the indicated durations after 24-h medium replacement with DMEM containing 10% FBS stripped of thyroid hormone by treatment with AG1X-8 resin (Sigma) and charcoal (Sigma).

Adenoviral Transduction—Adenoviruses expressing non-specific scrambled short hairpin RNA (shRNA) or shRNA against Ncor1 mRNA were generated using a BLOCK-it adenoviral RNAi expression system following the manufacturer’s instructions (Invitrogen). shNcor1 (3910–3930) targeted the sequence 5’-CATCAAGGGCCATGTATTTC-3’. TaT1.1 cells were transduced 24 h after the cells were seeded with adenoviruses to knock down the gene(s) of interest. The medium was changed the following day, and cells were harvested 72 h after infection. Hormone treatment with T₃ was done using stripped serum 8 h before harvesting. The concentration of adenoviruses was determined and all solutions had equivalent titer. Scrambled adenovirus control was used in the same concentration as the virus of interest.

RNA Isolation and Quantitative PCR—Total RNA from TaT1.1 cells was extracted by standard methodology (TRIZol reagent; Invitrogen). One microgram of total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR analyses were performed in a fluorescent temperature cycler using SYBR Green reagent according to the recommendations of the manufacturer (Bio-Rad). Primers for Ncor1, Ncor2, Tshb, Cga, Gh, Nr1d1, and 36B4 are listed in supplemental Table S1. Cycle threshold values for 36B4 were used to normalize each sample. All results are expressed as a fraction of samples treated with vehicle only.

Western Blot Analysis—TaT1.1 cells were treated with experimental conditions, after which whole cell extracts were prepared in 1× radioimmunoprecipitation assay buffer (Sigma) containing protease and phosphatase inhibitors (Roche Applied Science). Extract (20 μg of total protein) was run on 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membrane was probed using antibodies for the following proteins: NCOR1 (gift from A. Hollenberg, generated against the C-terminal portion of protein), SMRT (Affinity Bio), NR1D1 (Santa Cruz Biotechnology), and β-actin (Chemicon).

Chromatin Immunoprecipitation—ChIP assays were performed on TaT1.1 cells at ~90% of confluence using ChIP-IT Express kit (Active Motif, Carlsbad, CA) following the manufacturer’s instructions. Immunoprecipitation was performed at 4 °C overnight. Antibodies against the following proteins were used from the indicated suppliers: rabbit IgG (Santa Cruz Biotechnology), NCOR1 (Abcam), acetyl-H3 (Upstate Biotechnology), acetyl-H4 (Upstate), and Rev-Erbα (Abcam). Cross-links were reversed, and the DNA was purified using QIAquick PCR Purification Kit (Qiagen). The DNA recovered from the assay was subjected to qPCR using specific primers designed to detect enrichment in the proximal promoters of Tshb, Cga, and Gh (supplemental Table S2).

Cell Synchronization—TaT1.1 cells were seeded overnight in 6-cm dishes in DMEM supplemented with 10% FBS and antibiotics. Culture medium was replaced with serum-free DMEM for 4 h. Cells were then shocked with DMEM containing 50% horse serum for 2 h, after which medium was replaced with 10% stripped FBS (t = 0 h). Cells were harvested using TRIZol and stored at −80 °C until analyzed for RNA.

Statistical Analysis—Values are represented as mean ± S.E. of at least three independent experiments, unless otherwise noted. Statistical testing was performed using an unpaired two-tailed Student’s t test. A p value of < 0.05 was considered significant, and the respective levels of significance and group sizes are stated in respective figure legends.

RESULTS

Thyroid Hormone-responsive Genes Are Differentially Regulated by NCOR1—Studies have shown that T₃ regulates thyrotopin subunit gene expression in the TaT1.1 thyrotoph cell line. Whereas T₃ reduces Tshb mRNA levels, it has no effect on Cga mRNA levels. Negative regulation of transcription by nuclear receptors is often associated with recruitment of corepressors, and NCOR1 is thought to be the key corepressor that regulates the THR. To test the importance of NCOR1 on gene expression in TaT1.1 cells, we treated cells with adenovirus expressing NCOR1 shRNA (shNcor1). Ncor1 mRNA levels were reduced 45%, whereas Ncor2 mRNA levels that encode a closely related transcription factor, SMRTs (silencing mediator of retinoic or thyroid hormone receptors), were unaffected (Fig. 1A). Western blot analysis confirmed depletion of NCOR1 protein in these cells (Fig. 1B).

Cga expression in TaT1.1 cells was not regulated by T₃, and there was no change after treatment with shNcor1 (Fig. 1C). Throughout this work, we use Cga as a negative control. Gh, a gene up-regulated by T₃ binding to THR, is generally repressed
by NCOR1 (27). TαT1.1 cells express Gh at low levels such that this gene can be used as a positive control in our experiments. Knockdown of NCOR1 not only raised basal expression but also resulted in a reduction in T₃-mediated activation of Gh (Fig. 1D). Although shNcor1 did not affect T₃ repression of Tshb mRNA levels, the baseline level (vehicle treatment) did increase significantly compared with treatment with scrambled shRNA (Fig. 1E).

**NCOR1 Regulation of Tshb Is Independent of T₃**—Given that knockdown of NCOR1 affects basal expression of Tshb, we next explored whether the T₃ response is altered by determining a T₃ concentration response after SC and shNcor1 treatment.
changes in acetylated H4 were observed in mRNA expression, but not T3-mediated levels, we next determined whether NCOR1 interacts directly with Tshb. We performed ChIP-qPCR, scanning for NCOR1 enrichment around the transcription start site of Tshb (Fig. 2). Our laboratory has previously published that THRs enrich at sites −1861 and +18 bp relative to the transcription start site, regions containing several TRE half-sites (26). NCOR1, however, was found to be enriched at a different site, −2959 bp, and its occupancy was unaffected by T3 treatment (Fig. 2). On the Gh gene, THR is bound to a −220 bp site (28, 29). NCOR1 was enriched in this region and released upon T3 treatment, consistent with reports on Gh regulation (Fig. 2). We expected this response on Gh because NCOR1 recruitment is reported to be mediated through binding to THR-β (THRβ). The contrasting action on Tshb lead us to hypothesize that NCOR1 may be bound by a different transcription factor than THRβ.

TRH, but Not T3, Affects Acetylation of Histone H3 on the Tshb Promoter—Activation of gene expression is associated with histone acetylation, whereas repression is usually associated with deacetylation. Following this dogma, we assessed the enrichment of acetylated histones on the Tshb promoter. In Fig. 3A, a ChIP assay shows no significant changes in histone H3 acetylation on Tshb or Cga after treatment with 10 nM T3. TRH is known to stimulate Tshb expression, and this response was confirmed by increased acetyl-H3 after hormone treatment at sites centered on −684 and +18 bp (Fig. 3B). The latter region corresponds to a region also enriched with THRβ (26). No changes in acetylated H4 were observed in Tshb or Cga after treatment with vehicle, T3, or TRH (Fig. 3, C and D). These findings indicate that the assembly of transcription factors that regulate Tshb is likely to contain both a T3-independent mechanism that uniquely involves NCOR1 and a TRH-dependent mechanism that is associated with histone acetylation. The interplay of these pathways was evidenced after knocking down NCOR1. Treating cells with shNcor1 resulted in a marked increase in acetyl-H3 at the +18 bp site (Fig. 3E). This study was performed without hormone treatment and could possibly be the explanation for the increase in baseline Tshb mRNA levels after shNcor1 transduction. For example, if more H3 is acetylated on Tshb, one would expect an increase in basal expression.

Tshb mRNA Expression Oscillates in a Thyrotroph Cell Line—To define further the hormone-independent mechanism of Tshb expression, we studied gene expression over time in TαT1.1 cells. Although the cyclic secretion of TSH expression may be critical to cellular homeostasis, little is known about the overall impact of this physiologic event or the mechanism mediating the rhythm. By synchronizing TαT1.1 cells in culture, we were able to mimic Tshb mRNA cycling in this pituitary cell line. According to established protocols to study circadian regulation, we synchronized TαT1.1 cells by incubating them in serum-free medium, followed by a brief period of serum shock in 50% horse serum, and then harvesting mRNA at various time points for a qPCR analysis (Fig. 4). In this model, Tshb mRNA levels cycled with a 36-hour period, with peaks at 18 and 54 h, and nadirs at 0 and 36 h. Because expression ampli-

**FIGURE 3.** TRH, but not T3, affects histone acetylation of TSH subunit genes. ChIP-qPCR was performed to scan acetylation of histone H3 (A and B) or H4 (C and D) to a region surrounding the Tshb, Gh, and Cga promoters in TaT1.1 cells treated with vehicle (Veh), 10 nM T3 (A and C), or 100 μM TRH (B and D). E, cells were treated with scrambled shRNA (SC) or Ncor1 shRNA (shNcor1) and harvested 72 h after transduction for ChIP-qPCR analysis on the Tshb promoter. The x axis shows the location of the forward primer relative to the transcription start site. Results are expressed as relative fold enrichment ± S.E. compared with background enrichment. **, p < 0.01 versus vehicle.
In each successive cycle, the amplitude of the circadian rhythm was dampened, as indicated by the decreasing levels of mRNA for Tshb. Despite this, mRNA levels for Nr1d1, a heme receptor transcribed on the antisense strand of Thra, showed a significant oscillation (Fig. 4). Initial Nr1d1 mRNA levels decreased, followed by an increase at 12 h, with peaks at 36 and 72 h, and a nadir at 54 h. NR1D1 protein levels followed a similar pattern (data not shown). When comparing these mRNA levels, Tshb levels began to decline after the 18-h peak, whereas the Nr1d1 levels increased. From 30 to 54 h, Nr1d1 levels declined, and Tshb mRNA levels increased. The second decline in Tshb mRNA levels (54 to 72 h) was again marked with an increase in Nr1d1. The two mRNA oscillations appeared to oppose each other.

Furthermore, NR1D1 and NCOR1 were jointly recruited to additional sites on Tshb (Fig. 5). This suggests that NR1D1 acts as a repressor of Tshb expression.

NR1D1, a heme receptor transcribed on the antisense strand of Thra, is a potent repressor of gene transcription and a key driver of circadian rhythms and metabolism. NR1D1 has also been associated with NCOR1-dependent repression of nuclear receptors, and direct binding between the two proteins has been established (11). We decided to investigate Nr1d1 expression in our synchronized Tα/H9251 T1.1 cells and also observed a significant oscillation in expression (Fig. 4). Initial Nr1d1 mRNA levels decreased before rising at 12 h, with peaks at 36 and 72 h, and a nadir at 54 h. NR1D1 protein levels followed a similar pattern (data not shown). When comparing these mRNA levels, Tshb levels began to decline after the 18-h peak, whereas the Nr1d1 levels increased. From 30 to 54 h, Nr1d1 levels declined, and Tshb mRNA levels increased. The second decline in Tshb mRNA levels (54 to 72 h) was again marked with an increase in Nr1d1. The two mRNA oscillations appeared to oppose each
NR1D1 and NCOR1 Co-localize to the Tshb Promoter—NR1D1 is an orphan nuclear receptor with a unique structure, in that it lacks the helix 12 motif that is typically required for coactivators to bind the receptor (30). Previously described as a DNA-binding protein that interacts with NCOR1, we hypothesize that NR1D1 regulates Tshb basal rhythm via recruitment of NCOR1. To study the interaction of these two proteins on DNA, we performed ChIP assays, scanning for NR1D1 and NCOR1 enrichment on the Tshb promoter (Fig. 5). When Nr1d1 expression is low at 4 h, it enriches at −2959 bp, but after 8 h, when NR1D1 levels are rising, additional enrichment at −2959 bp and a new enrichment at −2362 bp are noted (Fig. 5A). NCOR1 recruitment to the Tshb promoter showed a similar pattern (Fig. 5B). Previously, we only observed NCOR1 enrichment at the −2959 site, but 8 h post-synchronization, NCOR1 is additionally recruited to this new −2362-bp region.

An NR1D1 Agonist Promotes Tshb Repression—Our data show that NCOR1 binds directly to the proximal promoter of Tshb and represses its basal mRNA expression independent of thyroid hormone regulation. NR1D1 also influences basal regulation of Tshb mRNA levels, likely through its recruitment of NCOR1 to the promoter. To confirm that NR1D1-based repression of Tshb is dependent on NR1D1, an NR1D1 agonist was employed (SR9011). SR9011 is a potent agonist of NR1D1 and demonstrates minimal cross-reactivity with other nuclear receptors (31). TαT1.1 cells were treated with increasing concentrations of SR9011 (or vehicle) for 8 h, and mRNA was harvested for qPCR studies. SR9011 induced a concentration-dependent decrease in Tshb mRNA levels (Fig. 6, A and B). One mM T3 and 5 μM SR9011 treatments resulted in maximal inhibition of mRNA expression of 65% and 80%, respectively. High concentrations of either ligand had no significant effect on Cga mRNA levels in this cell line (data not shown). ChIP assays showed that addition of 5 μM SR9011 to TαT1.1 cells also promoted NCOR1 recruitment to the −2362-bp region of Tshb in addition to the −2959-bp site (Fig. 6C). In summary, these data confirm our hypothesis that NCOR1 is recruited to NR1D1, triggering repression of Tshb gene expression (Fig. 7).

DISCUSSION

For decades, researchers have studied the role of thyroid hormone in the regulation of metabolism. On positively regulated genes, T3 binds to THR, inducing a conformational change that favors coactivator over corepressor binding. The regulation of genes repressed by thyroid hormone, however, is not a simple reversal of this process. Thyroid hormone repression is critical for regulation of the HPT axis and hence thyroid hormone synthesis. TSH is central to regulation of the HPT axis, and both subunit genes that comprise TSH are down-regulated by T3 in vivo (29, 32). We have previously shown that T3-bound THRβ represses Tshb subunit gene expression by recruiting cofactors to the ligand-binding domain (33). Our current findings show that there are two pathways for Tshb repression: one that involves T3 and THR and one that involves NCOR1 independent of T3. The latter pathway interested us in that it suggested a relationship between NCOR1 and NR1D1 on Tshb that has not been described previously.

Our first objective in establishing the role of NCOR1 on T3-responsive genes was to knock it down using an shRNA adenoviral construct. Knockdown of NCOR1 on the thyroid hormone positively regulated Gh gene demonstrated that NCOR1 was dismissed from the proximal Gh promoter as suggested previously. In the absence of T3, cells treated with shNcor1 displayed elevated basal Gh mRNA expression and eliminated any further increase after T3 treatment. On the negatively regulated Tshb, however, this increase in basal expression did not hinder T3 repression. In fact, comparing an extensive T3 concentration response between scrambled and
shNcor1 treatments revealed absolutely no change in the relative T3 inhibition of Tshb mRNA levels.

In this regard, our findings are in agreement with in vivo models of Ncor1 action. Although global deletion of Ncor1 is embryonic lethal, mutating the inhibitory domains of Ncor1 revealed a hypersensitivity to TH in peripheral tissues that was not seen in the HPT axis (10, 34). This is likely due to changes in basal gene expression that we also find in the current investigation using a thyrotriph cell line. Because of its critical role in controlling T3-mediated development, metabolism, and other vital processes in the body, TSH secretion requires a highly conserved and tightly regulated system of control. The T3 negative feedback response seems to be protected at the level of the pituitary and is not affected by Ncor1. A T3-independent Ncor1 pathway was further confirmed when ChIP assays showed enrichment of Ncor1 binding to the Tshb promoter sites, which are >1000 bp upstream of reported THR binding sites (26). Ncor1 was neither released nor further enriched at these sites after treatment with T3.

Because acute repression of TSH by T3 was unaffected by Ncor1 depletion, we next evaluated whether baseline Tshb mRNA expression was affected by Ncor1 depletion. A well-known characteristic of TSH and T3 secretion are their circadian secretory patterns. Metabolic processes can be affected by oscillations in hormone levels, light/dark periodicity, and feeding and are perpetuated by a core clock in the suprachiasmatic nucleus of the hypothalamus. BMAL1-CLOCK, the heterodimeric transcriptional controller of this central clock, is a direct target of NR1D1, and together, are required for the onset and continuation of each period in the cycle (16, 19). We explored the involvement of NR1D1 in our model because of its known role in circadian rhythm generation, as well as its direct interaction with Ncor1.

When the TaT1.1 thyrotriph cell line was synchronized, we were able to mimic a circadian cycle of Tshb mRNA levels in these cells. The mRNA expression of Tshb and Nr1d1 displayed opposing cycles, with peaks for one matching the nadir for the other, and vice versa. If Nr1d1 were truly a mediator of Ncor1-driven Tshb repression, we would need to demonstrate its direct binding to Tshb, as well as interaction with Ncor1. Nr1d1 did show enrichment to Tshb at the same locus as Ncor1. In addition, at a time when Nr1d1 protein expression was increasing, it was enriched at a second site not described previously. Using the Nr1d1 agonist SR9011, we showed that activation of Nr1d1 was sufficient to elicit Ncor1 binding at the two sites we identified and lead to a reduction in Tshb mRNA levels.

Acute control of TSH secretion is tightly regulated through thyroid hormone negative feedback. We believe that basal regulation of Tshb is regulated by Nr1d1, which recruits Ncor1 and leads to transcriptional repression. Recent studies have revealed a dynamic balance and highly protected role of Nr1d1 in circadian and metabolic regulation (17). Conserving its activity on multiple levels demonstrates its vital importance and necessity in not just maintaining, but also driving, the core clock. Given that Nr1d1 also drives circadian regulation of Tshb expression by binding to the promoter and recruiting Ncor1, it may provide a previously unknown way for metabolism to be regulated in a circadian rhythm by T3. Our work opens a new line of investigation linking NR1D1, circadian TSH and T3 secretion, and metabolism.

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