Thyroxine-induced expression of pyroglutamyl peptidase II and inhibition of TSH release precedes suppression of TRH mRNA and requires type 2 deiodinase

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Abstract

Suppression of TSH release from the hypothyroid thyrotrophs is one of the most rapid effects of 3,3′,5′-triiodothyronine (T3) or thyroxine (T4). It is initiated within an hour, precedes the decrease in TSHβ mRNA inhibition and is blocked by inhibitors of mRNA or protein synthesis. TSH elevation in primary hypothyroidism requires both the loss of feedback inhibition by thyroid hormone in the thyrotrophs and the positive effects of TRH. Another event in this feedback regulation may be the thyroid hormone-mediated induction of the TRH-inactivating pyroglutamyl peptidase II (PPII) in the hypothalamic tanycytes. This study compared the chronology of the acute effects of T3 or T4 on TSH suppression, TRH mRNA in the hypothalamic paraventricular nucleus (PVN), and the induction of tanycyte PPII. In wild-type mice, T3 or T4 caused a 50% decrease in serum TSH in hypothyroid mice by 5 h. There was no change in TRH mRNA in PVN over this interval, but there was a significant increase in PPII mRNA in the tanycytes. In mice with genetic inactivation of the type 2 iodothyronine deiodinase, T3 decreased serum TSH and increased PPII mRNA levels, while T4-treatment was ineffective. We conclude that the rapid suppression of TSH in the hypothyroid mouse by T3 occurs prior to a decrease in TRH mRNA though TRH inactivation may be occurring in the median eminence through the rapid induction of tanycyte PPII. The effect of T4, but not T3, requires the type 2 iodothyronine deiodinase.

Introduction

The feedback regulation of TSH secretion by thyroid hormones is mediated by the binding of 3,3′,5′-triiodothyronine (T3) to the TRβ2 receptor in the pituitary and hypothalamus (Chiamolera & Wondisford 2009). The acute suppression of TSH release from the pituitary is initiated within 30 min of the injection of thyroxine (T4) or T3 to the hypothroid rat and the degree of suppression parallels that of the saturation of the nuclear T3 receptors in the pituitary (Silva & Larsen 1977, Larsen et al. 1981). This response is blocked by inhibitors of mRNA or protein synthesis, suggesting that there may be induction or suppression of specific proteins that are required for this effect (Bowers et al. 1968a,b, Vale et al. 1968). The decrease in circulating TSH in these models occurs well before suppression of the synthesis of TSHβ mRNA and, in fact, studies have shown that in the early phases of the response, there is an increase in pituitary TSH content, suggesting that synthesis proceeds for some period of time in association with inhibition of release of the hormone (Silva & Larsen 1978, Shupnik et al. 1989). While most studies have focused on events in the pituitary, it is clear that positive regulation of TSH by TRH is also required for TSH elevation during hypothyroidism, indicating that this is not simply due to the absence of negative feedback regulation on the thyrotroph TSH (Nikrotdhanond et al. 2006).

It is well known that the conversion of T4 to T3 by the type 2 deiodinase (D2) is required for suppression of TSH at the pituitary level (Larsen et al. 1979). The requisite degree of saturation of the thyrotroph nuclear T3 receptors requires both circulating T3 and D2-mediated conversion of intracellular T4. The normal feedback regulation of TRH mRNA synthesis similarly requires both circulating T3 and that derived from D2-mediated T4 to T3 conversion.
(Kakucska et al. 1992, Lechan & Fekete 2005). Since there is no D2 mRNA in the paraventricular region of the hypothalamic paraventricular nucleus (PVN) where hypophysiotropic TRH is synthesized, it has been proposed that the D2 highly expressed in tanycytes of the mediobasal hypothalamus may provide T3 to TRH-producing neurons through extensive cell to cell interactions (Riskind et al. 1987, Tu et al. 1997, Fekele & Lechan 2007). Recent evidence has suggested that inactivation of TRH in the median eminence by pyroglutamyl peptidase II (PPII), a membrane-bound, highly specific TRH peptidase expressed in the tanycyes (Charli et al. 1998, Heuer et al. 2000), contributes to central regulation of TSH secretion (Sanchez et al. 2009). PPII is also expressed in lactotrophs and somatotrophs in the rat pituitary but not in thyrotrophs (Heuer et al. 1998, Cruz et al. 2008), indicating that pituitary PPII may not be involved in TSH regulation. As PPII synthesis is a T3-dependent process, with PPII mRNA being induced in the median eminence within a few hours of exposure to T3 or T4 (Sanchez et al. 2009), it suggests that its acute effects to inactivate TRH are localized in this region.

The D2 knockout mouse is resistant to the feedback effects of T4 on pituitary TSH release in both the euthyroid and the hypothyroid states (Schneider et al. 2001, Christoffolete et al. 2007). In this study, we used this model to analyze the chronological correlations between the inhibition of TSH release in response to T4 or T3 in intact animals with the induction of PPII mRNA in the tanycyes as opposed to preproTRH mRNA in the PVN. Our results indicate that the increase in PPII mRNA occurs before any reduction in intact preproTRH mRNA, suggesting that an increase in PPII with its consequent inactivation of TRH could play a role in the acute suppression of TSH release by T3 or T4.

Materials and Methods

Animals

All animal experimental protocols were approved by the Animal Research Committee of Harvard Medical School. C57BL/6j mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). D2KO/C57BL/6j are as described previously (Schneider et al. 2001). All animals were maintained under 12 h light:12 h darkness cycle and the standard animal facility temperature and humidity.

Hypothyroidism induction and experimental protocol

Mice were made hypothyroid by placing them on drinking water containing 0.1% MMI (Sigma) and 1% KClO₄ (Fisher Scientific Co., Pittsburgh, PA, USA; MMI/KClO₄), as described previously (Marsili et al. 2010). On the day of the experiment, a serum sample was obtained from the tail for TSH measurement. Immediately after, 6n-propylthiouracil (PTU; 2 mg/animal) was administered intraperitoneally to block D1 activity. One hour later, D2KO and WT mice were subdivided into three groups, each mouse receiving a single i.p. injection of T4 (3 μg/100 g body weight), T3 (1-2 μg/100 g body weight), or vehicle (PBS). Five hours after the hormone/vehicle administration, the mice were killed with an isoflurane overdose and blood collected via cardiac puncture.

Tissue processing

Animals were overdosed with pentobarbital (50 mg/kg; Ovation Pharmaceuticals, Inc., Deerfield, IL, USA) and perfused transcardially with 0.01 M PBS (pH 7.4) containing 150 000 U/l heparin sulfate, followed by 4% paraformaldehyde in PBS. The brains were removed and postfixed by immersion in the same fixative for 2 h at room temperature. Tissue blocks containing the hypothalamus were cryoprotected in 25% sucrose/PBS at 4 °C overnight and then snap frozen on dry ice. Serial 18 μm-thick coronal sections through the rostrocaudal extent of PVN and median eminence were cut on a cryostat (Leica CM3050 S; Leica Microsystems, Wetzlar, Germany) and adhered to Superfrost/Plus glass slides (Fisher Scientific Co.) to obtain four sets of slides, each set containing every fourth section through the PVN or median eminence. The tissue sections were desiccated overnight at 42 °C and stored at ~80 °C until prepared for in situ hybridization histochemistry.

In situ hybridization histochemistry

Every fourth section through the PVN or median eminence was hybridized with an 800 bp single-stranded [35S]UTP-labeled cRNA probe complementary to the entire coding region of the mouse TRH gene, or 644 bp single-stranded [35S]UTP-labeled cRNA probe complementary to the coding region of rat PPII (nucleotides 129–773), respectively, as described previously (Sanchez et al. 2009, Kadar et al. 2010). Hybridizations were performed under plastic coverslips in a buffer containing 50% formamide, a twofold concentration of standard sodium citrate (2X saline sodium citrate), 10% dextran sulfate, 0.25% BSA, 0.25% Ficol 400, 0.25% polyvinylpyrrolidone 360, 250 mM Tris (pH 8.0), 0.5% SDS.

Table 1 Serum thyroid hormone concentrations in hypothyroid WT and D2KO mice 5 h after hormone or vehicle injection (mean ± s.e.m.; n=5–7 per group)

<table>
<thead>
<tr>
<th></th>
<th>Serum T3 (ng/ml)</th>
<th>Serum T4 (μg/dl)</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>D2KO</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
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</tr>
<tr>
<td>Vehicle</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>T3 (1.2 μg i.p./100 g bw)</td>
<td>19.4±4.0*</td>
<td>16.3±3.0*</td>
</tr>
<tr>
<td>T4 (3 μg i.p./100 g bw)</td>
<td>0.6±0.1</td>
<td>0.4±0.2</td>
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</tbody>
</table>

*P<0.001 versus vehicle.
250 μg/ml denatured salmon sperm DNA, and $5 \times 10^5$ c.p.m. of the radiolabeled probe for 16 h at 55°C. Slides were dipped into Kodak NTB autoradiography emulsion (Eastman Kodak) diluted 1:1 in distilled water and the autoradiograms developed after 3 d of exposure for TRH mRNA or 30 d of exposure for PPII mRNA at 4°C. The specificity of hybridization was confirmed using sense probes, which resulted in the total absence of specific hybridization signal in the hypothalamus.

**Image analysis**

Slides were visualized with an Axioplan 2 imaging microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA) under dark-field illumination using a COHU 4912 video camera (COHU, Inc., San Diego, CA, USA), and the images analyzed with a Macintosh G4 computer using Scion Image software (National Institutes of Health, Bethesda, MD, USA). Background was removed by thresholding the image and integrated density values (density × area) of the hybridized regions were measured in rostrocaudal serial sections through the PVN or median eminence in one set of slides for each animal. Nonlinearity of radioactivity in the emulsion was evaluated by comparing density values with a calibration curve created from autoradiograms of known dilutions of the radiolabeled probes, immobilized on glass slides in 1.5% gelatin, fixed with 4% paraformaldehyde, and exposed and developed simultaneously with the in situ hybridization autoradiograms.

**Serum T₄, T₃, TSH measurement**

All hormones were measured by RIA after collecting blood from the tail vein. Serum T₄ and T₃ were measured using the COAT-A-COUNT total T₄ and T₃ kit (DPC, Los Angeles, CA, USA), following the manufacturer’s instructions, with mouse standard curves prepared in charcoal-stripped (T₄ and T₃ deficient) mouse serum as described previously (Christofolete et al. 2007, Marsili et al. 2010). TSH was determined using the rat TSH RIA from Alpco Diagnostic (Salem, NH, USA). All values fell within the linear range of a curve generated by the serial dilution of sample dilution buffer. The normal range for T₄ was 1.61 ± 0.17 and 2.79 ± 0.32 μg/dl for WT and D2KO respectively. The normal range for T₃ was 0.76 ± 0.07 and 0.77 ± 0.06 ng/ml for WT and D2KO respectively (Christofolete et al. 2007). TSH concentrations (ng/ml) were determined by extrapolating from the intercept of the high TSH mouse serum with the purified rat TSH standard curve supplied by the manufacturer, after correction for the difference of the nonspecific binding obtained with serum versus the nonspecific binding obtained with the assay.
buffer (Pohlenz et al. 1999). TSH concentrations were 4.04 ± 0.67 (range from 3.32 to 4.93) and 35.7 ± 5.2 (range from 27.5 to 47.8) ng rat equivalent/ml of rat equivalent serum in euthyroid and hypothyroid male mice respectively.

**Statistical analysis**

Results are presented as mean ± s.e.m. When only two groups were analyzed, statistical significance was determined using an unpaired Student’s t-test. Two-way ANOVA followed by Bonferroni correction using Prism 4 software (GraphPad Software, Inc., San Diego, CA, USA) was used to compare the effects of three different treatments on two genotypes (WT and D2KO). P values < 0.05 were considered statistically significant.

**Results**

Both wild-type and D2KO mice were markedly hypothyroid after 5 weeks of treatment with antithyroid drugs, as confirmed by measurements of T3, T4, and TSH (Table 1 and Fig. 1). Serum TSH values were markedly elevated in vehicle-treated mice in both groups in the range of 30–40 ng/ml, about tenfold the normal value (Fig. 1). T3 caused a marked increase in serum T3 5 h after injection in both WT and D2KO mice (Table 1). Serum T4 concentrations were also three- to four-fold the normal level in both wild-type and D2KO mice after the T4 injection, with no difference between the two genotypes. Since all animals had received PTU to block D1-mediated T4 to T3 conversion, it was not surprising that the serum T3 in the T4-treated mice was not significantly different from vehicle-treated mice. Injection of vehicle and manipulation of the animals caused no change in the serum TSH values (Fig. 1). T4 caused an ~60% decrease in TSH in the wild-type animals (P < 0.001) but had no effect in the D2KO mice (Fig. 1). On the other hand, both WT and D2KO animals had an ~70–80% decrease in TSH after injection of T3 (P < 0.001).

To determine whether the T3- or T4-induced acute decrease in TSH involved suppression of TRH mRNA, we performed in situ hybridization examination of preproTRH mRNA expression in the PVN of the same mice. In all animals, the TRH mRNA was markedly increased over that in euthyroid mice, as a consequence of their hypothyroidism (Kadar et al. 2010). Despite the significant decrease in TSH in all mice receiving T3 and in the WT mice receiving T4, there was no significant change in the integrated density values of the TRH mRNA in the thyroid hormone-treated groups (Fig. 2).

Because it has been previously shown that there is an increase in the PPII mRNA in hypothalamus 4–7 h after a single dose of T3 or T4 (Sanchez et al. 2009), we examined the mediobasal hypothalamus for changes in PPII mRNA. In vehicle-treated WT animals, PPII mRNA was barely detectable in tanyctyes lining the floor of the third ventricle but increased significantly in WT mice treated with T4 (Fig. 3). No change was observed in the T4-treated D2KO animals (Fig. 3). Thus, the tanyctyes in the median eminence require D2-mediated T4 to T3 conversion for induction of PPII transcription, and this occurs within a 5 h interval. As expected, PPII mRNA in both WT and D2KO mice responded modestly to T3 (Fig. 3) with a significant increase in PPII (P < 0.05), which was, however, less that that in the T4-treated WT animals (P < 0.01).

![Figure 3](https://example.com/fig3.png)
Discussion

These studies confirm a previous report that administration of both T4 and T3 results in >50% suppression of TSH in hypothyroid mice within 5 h and that the response to T4, but not to T3, is absent in D2KO mice (Schneider et al. 2001). This pattern is similar to the results found earlier in hypothyroid rats in which the degree of acute suppression of TSH release was shown to parallel the occupancy of the pituitary T3 receptors (Silva & Larsen 1977, 1978), an effect blocked by inhibition of D2 with iodoacetic acid (Larsen et al. 1979). A decrease in TSH secretion from TzT1 mouse thyrotrh cells by T4 also requires D2-mediated T4 to T3 conversion but does not require TRH (Christoffolete et al. 2007). Thus, some or all of the acute effects of T4 may be due to a direct interaction of the intracellular T3 produced by D2 in the thyrotrhons.

In addition, TRH synthesis is also negatively regulated by T3 (Segerson et al. 1987). While a decrease in preproTRH gene transcription by T3 has been demonstrated within 5 h using an intronic probe, we found no change in the TRH mRNA content of the hypothalamus over this time period (Fig. 2; Sugrue et al. 2010). This argues that a decrease in TRH synthesis is not required for the rapid inhibition of TSH release by thyroid hormone in the hypothyroid mouse but does not eliminate the possibility that an acute suppression of TRH also play a role in this response.

Recent studies have identified high expression of the TRH-inactivating PPII in the tanycytes in the floor of the third ventricle. This metalloprotease is encoded by a positively T3-responsive gene, and its mRNA increases as early as 4 h within the cell bodies of the tanycyte population (Sanchez et al. 2009). There is a close association between tanycyte end foot processes and preproTRH-containing axon terminals in the median eminence, suggesting a potential inactivation of TRH at this site. Our experiments revealed a significant increase in PPII mRNA within 5 h of T3 treatment, with an even more potent effect of T4 at this dosage in WT mice, while PPII expression was unchanged after T4 administration in D2KO mice. These results indicate that D2-mediated T4 to T3 conversion, presumably in tanycytes, is required for the rapid induction by T4 of PPII mRNA in hypothyroid mice. Of particular importance, however, is the observation that PPII inhibition increases the TSH response to TRH in thyrotrhons per se through an as yet undefined mechanism.

We cannot exclude the possibility that increase in somatostatin or dopamine could be involved in this acute response. These agents are known to inhibit TSH release and could be stimulated by T3. However, in vitro effects of these agents require several days rather than hours of exposure (Foord et al. 1984, Levy et al. 1992, Tam et al. 1996). Given the previously demonstrated blockade of this T3 effect by protein synthesis or gene transcription inhibitors (Bowers et al. 1968a,b, Vale et al. 1968), and the correlation between T3 receptor occupancy and the magnitude of the effect in earlier studies (Silva & Larsen 1977, 1978), a rapid gene transcriptional effect in thyrotrhons and perhaps in the tanycytes seems to be a more attractive hypothesis for the acute action of T3 in this system.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Institutes of Health Grants DK36256 and T32DK007529 to P R L and DK37021 to R M L A M was partially supported by a fellowship stipend from Department of Endocrinology and Kidney, University Hospital of Pisa, Italy.

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Received in final form 15 July 2011
Accepted 25 July 2011
Made available online as an Accepted Preprint 25 July 2011