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 hypothalamic 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.

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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 hypothyroid 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 (Nikrodhanond *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 parvocellular region of the hypothalamic paraventricular nucleus (PVN) where hypothypophysiotropic 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, Fekete & 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 tanycytes (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 tanycytes 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/ClO₄), 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 15 000 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, Nussloch GmbH, 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 (2× saline sodium citrate), 10% dextran sulfate, 0.25% BSA, 0.25% Ficoll 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)

	Serum T ₃ (ng/ml)		Serum T ₄ (µg/dl)	
	WT	D2KO	WT	D2KO
Treatment				
Vehicle	0.3 ± 0.1	0.2 ± 0.1	1.1 ± 0.2	0.9 ± 0.1
T ₃ (1.2 µg i.p./100 g bw)	19.4 ± 0.4*	16.3 ± 3.0*	1.2 ± 0.2	0.9 ± 0.1
T ₄ (3 µg i.p./100 g bw)	0.6 ± 0.1	0.4 ± 0.2	13.8 ± 2.5*	11.5 ± 2.5*

*P<0.001 versus vehicle.

Figure 1 Effect of T_3 and T_4 on serum TSH in hypothyroid WT and D2KO mice. TSH values were measured before and 5 h after i.p. administration of vehicle, T_4 (3 $\mu\text{g}/100$ g body weight) or T_3 (1.2 $\mu\text{g}/100$ g body weight). All animals received 2 mg PTU i.p. 1 h before hormone or vehicle administration. (A) Wild-type mice; (B) D2KO mice. Data are mean $\pm$ S.E.M. of five to seven animals per group; *** P < 0.001 for difference from pre-injection; NS, not significant.

250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, and 5×10^5 c.p.m. of the radiolabeled probe for 16 h at 55 $^\circ\text{C}$. 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 $^\circ\text{C}$. 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 $\times$ 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 T_4 , T_3 , TSH measurement

All hormones were measured by RIA after collecting blood from the tail vein. Serum T_4 and T_3 were measured using the COAT-A-COUNT total T_4 and T_3 kit (DPC, Los Angeles, CA, USA), following the manufacturer's instructions, with mouse standard curves prepared in

charcoal-stripped (T_4 and T_3 deficient) mouse serum as described previously (Christoffolete *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 T_4 was 1.61 ± 0.17 and 2.79 ± 0.32 $\mu\text{g}/\text{dl}$ for WT and D2KO respectively. The normal range for T_3 was 0.76 ± 0.07 and 0.77 ± 0.06 ng/ml for WT and D2KO respectively (Christoffolete *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

Figure 2 Absence of an acute response of *TRH* mRNA 5 h after T_3 and T_4 treatment in hypothyroid WT and D2KO mice. (A) Dark-field illumination photomicrographs of *TRH* mRNA in the medial parvocellular subdivision of the hypothalamic paraventricular nucleus (PVN) in hypothyroid WT (A–C) and D2KO mice (A1–C1) 5 h after i.p. administration of vehicle, T_4 (3 $\mu\text{g}/100$ g body weight) or T_3 (1.2 $\mu\text{g}/100$ g body weight). (D) Graph represents the densitometric analyses of the *in situ* hybridization autoradiograms. Data are analyzed by two-way ANOVA after Bonferroni correction. NS, not significant. Data are mean $\pm$ S.E.M. of five to seven mice per group. III, third ventricle.

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 $\pm$ 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 T_3 , T_4 , 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). T_3 caused a marked increase in serum T_3 5 h after injection in both WT and D2KO mice (Table 1). Serum T_4 concentrations were also three- to four-fold the normal level in both wild-type and D2KO mice after the T_4 injection, with no difference between the two genotypes. Since all animals had received PTU to block D1-mediated T_4 to T_3 conversion, it was not surprising that the serum T_3 in the T_4 -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). T_4 caused an $\sim 60\%$ 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 T_3 ($P < 0.001$).

To determine whether the T_3 - or T_4 -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 T_3 and in the WT mice receiving T_4 , 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 T_3 or T_4 (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 tanycytes lining the floor of the third ventricle but increased significantly in WT mice treated with T_4 (Fig. 3). No change was observed in the T_4 -treated D2KO animals (Fig. 3). Thus, the tanycytes in the median eminence require D2-mediated T_4 to T_3 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 T_3 (Fig. 3) with a significant increase in PPII ($P < 0.05$), which was, however, less than that in the T_4 -treated WT animals ($P < 0.01$).

Figure 3 The acute increase in PPII mRNA induced by T_4 , but not T_3 , is absent in D2KO mice. Dark-field illumination photomicrographs of PPII mRNA (arrows) in tanycytes lining the floor of the third ventricle (III) at the level of the median eminence (ME) in hypothyroid WT (A–C) and D2KO (A1–C1) 5 h after administration of vehicle, T_4 (3 $\mu\text{g}/100$ g body weight) or T_3 (1.2 $\mu\text{g}/100$ g body weight). (D) Graph represents the densitometric analyses of the *in situ* hybridization autoradiograms. Data were analyzed by two-way ANOVA after Bonferroni correction. *** $P < 0.001$; NS, not significant. Data are mean $\pm$ S.E.M. of five to seven mice per group.

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 nuclear T₃ receptors (Silva & Larsen 1977, 1978), an effect blocked by inhibition of D2 with iopanoic acid (Larsen *et al.* 1979). A decrease in TSH secretion from T α T1 mouse thyrotroph 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 thyrotrophs.

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 cold exposure or TRH (Sanchez *et al.* 2009), indicating that PPII plays a role in increasing endogenous TSH release. It is conceivable, therefore, that the acute fall of TSH in hypothyroid animals treated with thyroid hormone could also involve a reduction in the TRH supply to thyrotrophs as a result of its degradation in the median eminence by the rapid T₃-mediated induction of tanycyte PPII. This may supplement the effect of T₃-induced suppression of TSH release in the thyrotroph *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 thyrotrophs 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.

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