Prolactin Secretion in Mice with Thyrotropin-Releasing Hormone Deficiency

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The physiological roles of TRH in pituitary lactotrophs, particularly during lactation, remain unclear. We studied the prolactin (PRL) status, including serum PRL and PRL mRNA levels in the pituitary, in nonlactating and lactating TRH-deficient (TRH−/−) mice with a rescue study with thyroid hormone and TRH. We found that, as reported previously for male TRH−/− mice, neither the morphology of the lactotrophs, PRL content in the pituitary, nor the serum PRL concentration was changed in nonlactating female TRH−/− mice. However, concurrent hypothyroidism induced a mild decrease in the PRL mRNA level. In contrast, during lactation, the serum PRL level in TRH−/− mice was significantly reduced to about 60% of the level in wild-type mice, and this was reversed by prolonged TRH administration, but not by thyroid hormone replacement. The PRL content and PRL mRNA level in the mutant pituitary during lactation were significantly lower than those in wild-type mice, and these reductions were reversed completely by TRH administration, but only partially by thyroid hormone replacement. Despite the low PRL levels, TRH−/− dams were fertile, and the nourished pups exhibited normal growth. Furthermore, the morphology of the pituitary was normal, and high performance gel filtration chromatography analysis of the PRL molecule revealed no apparent changes. We concluded that 1) TRH is not essential for pregnancy and lactation, but is required for full function of the lactotrophs, particularly during lactation; and 2) the PRL mRNA level in the pituitary is regulated by TRH, both directly and indirectly via thyroid hormone. (Endocrinology 147: 2591–2596, 2006)

TRH IS WELL KNOWN to be a major regulator of TSH synthesis and secretion in the anterior pituitary, but TRH has also been reported to be a potential stimulator of prolactin (PRL) synthesis and secretion from the anterior pituitary (1). Exogenous administration of TRH has been shown to evoke a significant elevation of the serum PRL concentration in men (2, 3). It has also been reported that in vitro TRH stimulates PRL release from dispersed pituitary cell cultures and PRL-secreting cell lines and augments the in vitro TRH stimulation of PRL. Whether TRH has also been reported to be a potential stimulator of prolactin (PRL) synthesis and secretion from the anterior pituitary (1).

Many studies have therefore been conducted to determine the physiological roles of TRH in pituitary lactotrophs in vivo in the last 3 decades, including studies involving the immunoneutralization of TRH, measuring TRH using the push-pull method during lactation, and measuring the mRNA level of prepro-TRH after suckling stimulation (6–14). Several studies suggested that TRH might be important for suckling-induced PRL release, whereas some reports, to the contrary, noted the lack of a coordinate increase in TSH and PRL in vivo and the inability of antiserum to block suckling-induced PRL release.

We have recently generated TRH-deficient (TRH−/−) mice using homologous recombination in embryonic stem cells. In these mice, all repetitive copies of TRH progenitor sequences in prepro-TRH were deleted. The TRH-deficient mice were viable and fertile, and exhibited characteristic tertiary hypothyroidism, with an elevation of biologically inactive serum TSH and mild hyperglycemia (15). The male TRH−/− mice had normal morphology of the pituitary lactotrophs, normal serum PRL concentration, and low PRL mRNA levels in their pituitaries. In contrast, TRH receptor subtype 1-deficient mice (TRH-R1) generated recently by Rabeler et al. (16) showed a different phenotype, with a low serum PRL level and low PRL mRNA levels in the pituitary, even in male and nonlactating female mice.

In the present study, taking advantage of TRH-deficient mice, we conducted experiments, including a rescue study with TRH, that could not be performed in receptor knockout mice. We found that TRH is required for the function of lactotrophs, particularly during lactation, and that TRH and thyroid hormone each significantly affect PRL production.

Materials and Methods

Animals

The procedures of animal care and use in this study were approved by the review committee on animal use at Gunma University (Maebashi, Japan). Animals were maintained on a 12-h light, 12-h dark schedule (lights on at 0600 h) and given laboratory chow and tap water ad libitum. All experiments were carried out between 0900–1100 h.

The mice with T4 replacement received 1.2 μg T4/100 g body weight, sc, for 10 d before the experiment. TRH replacement in TRH−/− mice was achieved by administering 1.0 μg/kg/14 d through a continuous miniosmotic pump over a period of 14 d.

For implantation of the osmotic pump, a small incision was made in the skin between the scapulae after the mice were anesthetized with diethyl ether. Using a hemostat, a small pocket was formed by spreading the sc connective tissue. The pump (Alzet model 2002, Alza Corp., Palo Alto) was then inserted into the pocket.
Confirm the results of Northern blot analysis, we performed real-time PCR with TaqMan probe (TaqMan Gene Expression Assays, Assay ID Mm00599949, Applied Biosystems, Foster City, CA) and cDNAs reverse transcribed from 200 ng total RNA (GeneAmp EZ rTth RNA PCR Kit, Applied Biosystems) using Applied Biosystems 7500. The results obtained from both procedures were well correlated (i.e. Northern blot analysis of male TRH−/− pituitary PRL mRNA, 63.4 ± 7.7% of the control; real-time PCR, 51.2 ± 5.1% of the control (n = 4)). To enable comparison of the present results with the previously published findings and to examine the quality of the RNA of each sample, we used Northern blot analysis throughout this study.

**Chromatographic characterization of PRL in TRH-deficient pituitary**

The elution patterns of PRL in pituitary extract from wild-type and TRH-deficient mice were analyzed with high-performance gel filtration chromatography (HPGFC; Zorbax GF-250 column, 9.4 × 250 mm; DuPont Co., Wilmington, DE). The column was equilibrated with phosphate buffer. A 50-µl sample was loaded on the column, the column was eluted with phosphate buffer at a flow rate of 1.0 ml/min, 30-ml fractions were collected, and the PRL content in each fraction was measured by RIA as described above.

**Statistical analysis**

Statistical analysis was performed using ANOVA and Duncan’s multiple range test. All values are expressed as the mean ± SEM.

**Results**

**Serum PRL level and pituitary PRL content did not differ between nonlactating wild-type and TRH−/− female mice**

As reported previously, the serum PRL level in male TRH−/− mice did not differ from that in wild-type mice (15). Therefore, we first examined the serum PRL level in nonlactating female mice. To exclude the effect of the menstrual cycle, the serum PRL level was measured during the preestrous period, because it has been reported that TRH mostly affects PRL secretion from the pituitary (1, 18). Although serum PRL levels in female mice were significantly higher than those in male mice, the values in nonlactating female TRH−/− mice were similar to those in wild-type mice [wild-type, 22.5 ± 4.4 ng/ml (n = 11); TRH−/−, 22.6 ± 2.7 ng/ml (n = 7); Fig. 1A].

We next compared the PRL content in mutant pituitary to that in wild-type pituitary. Reflecting the serum PRL level, the PRL content in the mutant pituitary was slightly, but not significantly, increased compared with that in wild-type mice, as shown in Fig. 1B [wild-type, 69.2 ± 12.0 ng/pituitary (n = 11); TRH−/−, 78.0 ± 22.0 ng (n = 8)].

**Decrease in PRL mRNA level in female TRH−/− pituitary due to concurrent hypothyroidism**

Although male TRH−/− mice showed a normal serum PRL concentration and normal PRL content in the pituitary, the pituitary PRL mRNA level in was significantly decreased due to hypothyroidism. Therefore, we examined the PRL mRNA level in female mice. Figure 2A shows representative data from Northern blot analysis, showing a single hybridization signal of PRL mRNA of approximately 1.0 kb. Similar to the decrease observed in male mice, a significant decrease in the PRL mRNA level (82.7 ± 6.5% of the control; n = 6; P < 0.05) was observed in female TRH−/− pituitary. However, this decrease was again reversed to normal levels by thyroid hormone replacement (105.6 ± 7.9 of the control; n =
indicating that the decrease in PRL mRNA in nonlactating TRH−/− female mice was also due to hypothyroidism rather than to the lack of TRH.

Therefore, all phenomena regarding PRL status observed in nonlactating female mice were similar to those in male mice. The only difference between male and female TRH−/− mice was that the degree of reduction in PRL mRNA in female mutant pituitary was milder than that in male mice (TRH−/− male, 63.4 ± 7.7% of the control; Fig. 2B).

**Significant decrease in serum PRL and pituitary PRL content in lactating TRH−/− mice**

Because the lack of TRH did not affect PRL status in male and nonlactating female mice, we next examined PRL status when the production of PRL was expected to be greatest; i.e. the serum PRL concentration and pituitary content were measured in lactating TRH−/− mice under continuous suckling (17). Compared with the concentration in nonlactating mice, the serum PRL concentration was markedly increased in both wild-type and TRH-deficient mice during lactation. However, the concentration in TRH-deficient mice was significantly lower than that in lactating wild-type mice, i.e. it was only 48% of the control (160.0 ± 15.2 ng/ml in mutant vs. 327.0 ± 37.0 in wild-type mice; n = 13 and 10, respectively; P < 0.01; Fig. 3A). To examine whether this reduction was due to hypothyroidism, we injected thyroid hormone daily for 10 d to achieve euthyroid status. However, this replacement did not alter the reduction (170.1 ± 18.0 ng/ml; n = 5),
suggesting that the decreased serum PRL concentration in lactating mice might be due to a direct effect of TRH deficiency. To verify this, we next performed prolonged TRH administration using an osmotic pump in TRH−/− mice. This treatment increased serum PRL to a completely normal level, as shown in Fig. 3A (304.0 ± 56.2 ng/ml; n = 5).

Furthermore, as shown in Fig. 3B, reflecting the reduction in serum PRL, the PRL content in lactating TRH−/− pituitary was markedly decreased to 54% of that in wild-type pituitary during lactation [2.07 ± 0.36 μg/pituitary in mutant pituitary (n = 6); 3.82 ± 0.42 in wild-type (n = 7); P < 0.01]. TRH replacement increased the PRL content in mutant pituitary to the normal level (3.80 ± 0.32 μg/pituitary; n = 4), but thyroid hormone replacement did not (2.02 ± 0.52 μg/pituitary; n = 5; P < 0.05). These data suggested that the reduction in serum PRL in lactating TRH-deficient mice might be due to the low level of synthesis of PRL resulting from the lack of TRH.

Pituitary PRL mRNA was regulated by both TRH and thyroid hormone during lactation

We next measured the PRL mRNA level in lactating TRH−/− pituitary and performed a rescue study with TRH and thyroid hormone. A representative Northern blot analysis is shown in Fig. 4A. Similar to the reduction in pituitary PRL content, the pituitary PRL mRNA level in mutant mice was significantly decreased to 50.3 ± 2.4% (n = 6; P < 0.01) of the level in wild-type pituitary. In contrast to the effect in nonlactating mice, thyroid hormone replacement only partially reversed this reduction, resulting in 72.1 ± 2.7% of the control PRL mRNA level (n = 6; P < 0.01 vs. wild-type; Fig. 4B). However, prolonged TRH administration using an osmotic pump led to complete recovery of the PRL mRNA level in TRH−/− pituitary (101.4 ± 3.0% of the control; n = 5). These findings clearly demonstrate that TRH directly affected the PRL mRNA level in lactating pituitary in addition to the indirect effect of TRH via thyroid hormone.

No morphological changes in pituitary lactotrophs or in the chromatographic profile of the pituitary PRL molecule in TRH−/− female mice

Because the production of PRL seemed to be decreased in lactating female mice, we next examined the morphology of pituitary lactotrophs using an antibody specific for mouse PRL.
PRL. Despite the lack of TRH, there were no apparent morphological changes in mutant lactotrophs compared with those in wild-type mice (data not shown). Furthermore, because the serum TSH level in TRH−/− mice showed a paradoxical increase with reduced biological activity, we analyzed the PRL molecule in TRH−/− pituitary using HPGFC. The chromatographic profiles of TRH−/− pituitary PRL were similar to those of PRL from wild-type pituitary, indicating that no apparent changes in the PRL molecule were induced by the lack of TRH (data not shown).

Normal growth of pups nourished by TRH−/− dams

If milk production was impaired by the low serum level of PRL in TRH−/− dams, the growth of pups nourished by them should be disturbed. Therefore, we next compared the growth curves of pups nourished by TRH−/− dams to those of wild-type mice. All pups were weaned 21 d after birth. As shown in Fig. 5, despite the low serum PRL level in TRH−/− dams, no significant changes in body weight were observed either before or after weaning, indicating that the low PRL level did not affect milk production sufficiently to alter the normal growth of the pups.

Discussion

We have demonstrated that TRH is not required for normal lactotroph function in male mice or nonlactating female mice. We previously reported significant alterations in the phenotypes of pituitary thyrotrophs in TRH-deficient mice, with decreases in staining intensity, number of thyrotrophs, TSH content, and TSH mRNA level in the pituitary, and demonstrated that these changes began during the early postnatal period (15, 17). These phenotypic changes led to an inappropriate postnatal increase in serum thyroid hormone and induced hypothyroidism within a few days after birth. Therefore, although TRH is known to be a strong stimulator of secretion and synthesis for both TSH and PRL, its role is completely different in thyrotrophs and lactotrophs. We concluded that TRH is not required for the embryonic development of either pituitary thyrotrphs or lactotrophs and is required for the postnatal development only of thyrotrphs.

Rabeler et al. (16) recently established TRH-receptor subtype 1 knockout mice (TRH-R1−/−). These mice showed similar phenotypes to TRH-deficient mice, including central hypothyroidism and impairment of PRL expression in TRH-R1−/− pituitary (16). However, the most striking difference between TRH−/− and TRH-R1−/− mice is that the serum PRL level was found to be low even in nonlactating females and males in TRH-R1−/− mice. Although the precise mechanism causing this difference remains unclear, several possibilities can be proposed. First, when we compared the thyroid hormone level in these mice, hypothyroidism was more severe in TRH-R1−/− mice, at approximately 40% of the wild-type mice, whereas it was about 60% in TRH−/− mice. Because, as demonstrated in this study, thyroid hormone significantly affects the pituitary PRL mRNA level, severe hypothyroidism may lead to a greater reduction of PRL synthesis in the TRH-R1−/− pituitary. Second, like other peptide hormones, TRH is cleaved from a large precursor peptide, prepro-TRH, generating several intervening peptides. Some intervening peptides of prepro-TRH have been reported to possess regulatory activity toward PRL synthesis, including the peptides corresponding to amino acids 160–169 and 191–199 of rat prepro-TRH (19–21). Because these intervening peptides were also disrupted in TRH−/− mice, this disruption may affect PRL status in the mutant mice. Third, TRH is known to be distributed throughout the entire brain, including the pituitary. Therefore, the lack of TRH in these regions may affect PRL production in the TRH−/− pituitary. Furthermore, a patient with TRH receptor mutations showed a normal serum PRL concentration with impairment of the response to TRH administration (22). Therefore, a genetic background other than TRH and TRH-R1 may also affect PRL production and secretion.

Despite the reduced PRL expression, neither lactation nor maternal behavior was severely impaired in TRH−/− mice, suggesting that there was enough PRL production to promote the synthesis and secretion of milk proteins in sufficient amounts in mammary alveoli. We examined the morphology of the mammary gland and found that there were no apparent abnormalities in branching and lobulation in mammary glands of TRH-deficient dams (data not shown). Thus, TRH-deficient mice did not exhibit any apparent impairment in reproductive functions, which was reported in PRL knockout and PRL receptor knockout mice (23–26).

Considering all the data from the present study, we propose the following hypothesis. In male and nonlactating female mice, the inhibitory effect of dopamine on PRL production and release may predominate over the stimulatory effect of TRH; however, thyroid hormone, which is regulated by TRH, may be able to affect PRL transcription. However, during lactation, the effect of dopamine is reduced to a level such that PRL production can be affected by TRH, and TRH and thyroid hormone, which is indirectly regulated by TRH, cooperatively stimulate PRL gene transcription, contributing to the full function of pituitary lactotrophs. Furthermore, Fjeldheim et al. (27) recently reported that continuous suckling induced increases in hypothalamic prepro-TRH mRNA and TRH-R1 mRNA levels in pituitary and hypothalamus,
but TRH-degrading enzyme mRNA levels were not changed in either pituitary or hypothalamus (27). Therefore, it is speculated that dynamic changes in TRH production and expression of TRH receptor and TRH-degrading enzymes may be involved in the full function of pituitary lactotrophs, particularly during lactation.

Acknowledgments

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