

Mechanism of Selective Retinoid X Receptor Agonist-Induced Hypothyroidism in the Rat

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The retinoid X receptor (RXR) agonist bexarotene can cause clinically significant hypothyroidism in cutaneous T cell lymphoma patients. The mechanism by which the RXR agonist produces this effect is unclear. We have studied the impact of a selective RXR agonist (rexinoid), LG100268, on rat thyroid axis hormones and show that the acute phase of hypothyroidism is associated with reduced pituitary TSH secretion. A single oral administration of LG100268 to naive Sprague Dawley rats causes a rapid and statistically significant decline in TSH levels (apparent in 0.5–1 h). Total T_4 and T_3 levels decline more gradually, reaching statistical significance 24 h after treatment. Increasing doses of LG100268 produce greater sup-

pression of thyroid axis hormones. To investigate the mechanism(s) mediating this suppression, we determined pituitary TSH β mRNA, TSH protein levels, and TRH-stimulated TSH secretion. Two hours after treatment, neither TSH β mRNA nor TSH protein levels were altered by LG100268. However, LG100268 treatment reduced the area under the curve for TRH-stimulated TSH secretion by 54%. We have identified an unexpected mechanism by which rexinoids induce hypothyroidism by acutely reducing TSH secretion from the anterior pituitary. This mechanism is independent of the rexinoid's previously demonstrated inhibition of TSH β gene transcription. (*Endocrinology* 143: 2880–2885, 2002)

THE RETINOID X receptor (RXR) is a member of the nuclear hormone receptor superfamily. RXR alters transcription after forming heterodimers with numerous nuclear hormone receptors, including peroxisome proliferator-activated receptors, retinoic acid receptors (RARs), thyroid hormone receptors, and the liver X receptor (1). In addition, RXRs may form homodimers (2) that are effective activators of transcription *in vitro* (3, 4). Selective RXR agonists (rexinoids), such as bexarotene (Targretin, formerly LGD1069) and LG100268, bind to RXR with high affinity (Table 1) and activate RXR homodimer-dependent transcription (5, 6). Bexarotene is effective in treating refractory, advanced stage cutaneous T cell lymphoma patients (7), and phase II clinical trial results indicate that it may prolong the survival of non-small cell lung cancer patients (8). In addition, both bexarotene and LG100268 lower plasma glucose levels in rodent models of type II diabetes mellitus (9, 10). Recently, we demonstrated that LG100268 suppresses hepatic glucose output and increases peripheral glucose disposal during euglycemic, hyperinsulinemic clamp studies in Zucker fatty rats (11).

Although selective RXR agonists have desirable therapeutic effects, side-effects have also been observed, including hypothyroidism (12). Patients receiving bexarotene treatment develop symptoms of hypothyroidism, with lowered circulating levels of pituitary TSH, T_4 , and T_3 . The suppression of thyroid axis hormones is reversible; the hormones return to normal levels upon cessation of the bexarotene treatment (12). The mechanism(s) for RXR agonist-induced

suppression of thyroid axis hormones is unclear, and understanding them may aid the development of next generation agents without this side-effect. In the present studies Sprague Dawley rats were treated with LG100268, and thyroid axis hormones were measured to investigate the mechanism(s) of RXR agonist-mediated suppression of the thyroid axis.

Materials and Methods

The protocols used in this study have been approved by the institutional animal care and use committee. Seven-week-old male Sprague Dawley rats (~200 g body weight) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The animals had free access to Purina 5008 diet (Ralston Purina Co., St. Louis, MO) and tap water, with a 12-h dark, 12-h light cycle (lights on from 0600–1800 h). Animals were acclimated in our facility for 5–6 d before treatment and were treated via oral gavage with 1 ml vehicle each day for 3 d before the experiment to acclimate them to the dosing procedure. The vehicle consists of 0.085% povidone (ISP Technologies, Inc., New Milford, CT), 1.5% lactose (Quest International, New York, NY), 0.026% Tween 80 (Sigma, St. Louis, MO), and 0.2% (v/v) Antifoam (Dow Corning Corp., Midland, MI).

On the day of the experiment, naive animals were treated with either vehicle alone or a suspension of LG100268 in the vehicle. Blood was collected via decapitation at the indicated times post treatment, and serum was prepared and kept frozen at –20 C until analyzed. Pituitaries were dissected and snap-frozen in liquid N_2 , then stored at –80 C for RNA or protein preparations.

TRH stimulation study

Two days before the experiment rats were cannulated with indwelling jugular vein cannulas as described previously (13). On the day of the experiment, animals were treated orally with vehicle or LG100268. Twelve-inch-long PE-50 tubes were connected to the cannulas to enable blood sampling without touching the animals. Two hours after treatment, blood samples (500 μ l) were collected via the cannulas for basal (time zero) TSH analysis. TRH (3 μ g/kg; Peninsula Laboratories, Inc., Belmont, CA) in saline with 0.1% BSA was injected via the cannulas, and

Abbreviations: all-trans-RA, All-trans-retinoic acid; AUC, area under the curve; 9-cis-RA, 9-cis-retinoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPL, lipoprotein lipase; RAR, retinoic acid receptor; RXR, retinoid X receptor.

TABLE 1. Receptor binding affinities for RXR ligands to RXR or RAR

Compound	RXR binding (K_d , nM)			RAR binding (K_d , nM)		
	α	β	γ	α	β	γ
All-trans-RA ^a	53	306	306	15	17	17
9-cis-RA ^a	8	15	14	93	97	148
Bexarotene ^b	34	78	78	>10,000	>10,000	>10,000
LG100268 ^a	3	3	3	>10,000	>10,000	>10,000

^a Historical data, Ligand Pharmaceutical, Inc., database.

^b Previously published data (26).

subsequent blood samples (500 μ l each) were collected at 15, 30, and 60 min after TRH injection. Lost blood volume was replaced with an equal volume of heparinized (10 U/ml) saline after each blood sampling. Plasma was prepared immediately and was kept frozen at -20 C until analyzed.

Northern blot analysis of TSH β mRNA

RNA was extracted from frozen rat pituitaries using Tri-Reagent (Sigma) according to the manufacturer's instructions. Briefly, each frozen pituitary was homogenized in 1 ml Tri-Reagent using a Tissue Tearor homogenizer (BioSpec Products, Inc., Bartlesville, OK). Samples were incubated at room temperature for 5 min, then 0.2 ml chloroform was added, and the samples were shaken. After centrifugation the aqueous phase was mixed with 0.5 ml isopropanol. Samples were again incubated for 5 min and centrifuged, and the liquid was removed. The RNA pellet was then washed with 70% ethanol and resuspended in water. RNA concentrations were determined by A_{260} measurements. Ten micrograms of rat pituitary total RNA were run on a 1% denaturing agarose gel and transferred by capillary blotting onto a nylon membrane (Hybond-N⁺, Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was prehybridized in the Rapid-hyb buffer (Amersham Pharmacia Biotech) at 65 C for 30 min. Hybridization was performed at 65 C for 1.5 h with a probe for the rat TSH β gene (102–304), that was labeled with ³²P by random priming (Stratagene, La Jolla, CA). After hybridization, the membrane was washed twice in 2 \times standard saline citrate and 0.1% sodium dodecyl sulfate for 15 min at room temperature and then incubated in 0.1 \times standard saline citrate and 0.1% sodium dodecyl sulfate for 20 min at 65 C. Autoradiography was performed on Kodak BioMax film (Rochester, NY) with an intensifying screen at -80 C. The Northern blot was quantified on a PhosphorImager Storm 860 (Molecular Dynamics, Inc., Sunnyvale, CA) and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal.

Hormone analysis

Total T₃ and T₄ were measured by RIA (Diagnostic Products, Los Angeles, CA). For T₃, 100- μ l serum samples were assayed. The assay range was 10–600 ng/dl, with a detection limit of approximately 7 ng/dl. Intra- and interassay variations ranged from 3.1–8.9% and 5.7–10.0%, respectively. For T₄, 25- μ l serum samples were assayed. The assay range was 5–240 ng/ml, with a detection limit of about 2.5 ng/ml. Intra- and interassay variations ranged from 2.8–3.8% and 4.2–14.5%, respectively. TSH was measured by RIA using a primary antibody to rat TSH (Amersham Pharmacia Biotech). Serum or plasma samples were assayed (100- μ l volume). The assay range was 1–64 ng/ml, with a detection limit of approximately 0.5 ng/ml. Intra-assay variation was about 4.8%, and interassay variation ranged from 8.5–16.2%.

To measure pituitary TSH content, frozen pituitaries were first thawed on ice and then dispersed in 100 μ l homogenization buffer by vortexing and pipetting. The homogenization buffer is made of 10 mM Tris (pH 7.4), 2 mM MgCl₂, 140 mM NaCl, 0.5 mM dithiothreitol, 0.1% Triton X-100, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and Complete Protease Inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Additional homogenization buffer (900 μ l) was added, and the tissues were further homogenized by pipetting. The extract was then cleared by centrifugation at 40,000 rpm for 20 min (Optima Ultracentrifuge, TLA 45 rotor, Beckman, Fullerton, CA), and the supernatant was analyzed using the TSH RIA kit. The results are expressed as micrograms of TSH per pituitary.

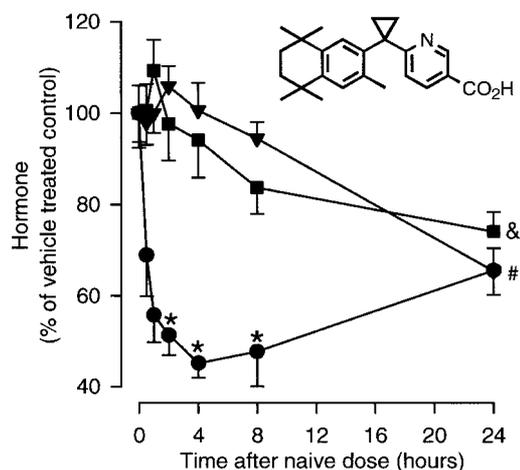


FIG. 1. TSH falls before T₃ or T₄. One group (n = 6) of animals was killed before treatment to determine basal (time zero) hormone levels. The remaining animals were orally treated with vehicle or LG100268 (3 mg/kg), and groups were killed at 0.5, 1, 2, 4, 8, and 24 h post treatment. The structure of LG100268 is shown in the upper right of the figure. Serum TSH, total T₄, and T₃ levels were analyzed using RIA. The hormone levels in LG100268-treated animals are expressed as a percentage of the mean for vehicle-treated rats killed at the same time. Data were statistically analyzed by *t* test. ●, TSH; ▼, total T₄; ■, total T₃. *, *P* < 0.05 vs. vehicle for TSH; #, *P* < 0.05 vs. vehicle for total T₄; &, *P* < 0.05 vs. vehicle for total T₃.

Statistical analysis

Data from the LG100268 dose response were analyzed using one-way ANOVA (Dunnett's method). Other data were analyzed by *t* test. The incremental area under the curves for TSH (AUCs above time zero TSH levels) was calculated on an individual animal basis for TRH-treated groups, followed by statistical analysis with *t* test.

Results

To study the kinetics of rexinoid effects on thyroid axis hormones, their levels were measured over 24 h after administration of LG100268 to naive Sprague Dawley rats. One group of animals (n = 6) was killed before treatment to determine basal (time zero) hormone levels. The rest of the animals were orally treated with either vehicle or LG100268 (3 mg/kg) and were killed at 0.5, 1, 2, 4, 8, and 24 h post treatment. The fall in TSH levels was apparent 30 min after LG100268 administration (Fig. 1), reached statistical significance 2 h after treatment, and continued to be significantly suppressed throughout the 2- to 8-h period after treatment (*P* < 0.05). Twenty-four hours after treatment, TSH levels in LG100268-treated animals partially recovered and were no longer statistically different from those in vehicle-treated animals. In comparison, there was no change in the levels of

either total T₃ or T₄ during the first 8 h after treatment (Fig. 1), but the levels of total T₃ and T₄ in LG100268-treated rats were significantly lower than those in vehicle-treated rats at the 24 h point ($P < 0.05$). These results demonstrate that LG100268 treatment first suppresses plasma TSH levels, which subsequently causes the decreases in circulating levels of both total T₃ and T₄.

Although TSH levels 24 h after a single administration of 3 mg/kg LG100268 are not statistically lower than those in vehicle-treated animals, larger doses of compound produce a statistically significant reduction at this time (Fig. 2). In addition, 24 h after a single oral administration of LG100268, total T₃ levels were significantly suppressed in rats treated with 10 or 30 mg/kg LG100268. Total T₄ was significantly suppressed by LG100268 at all three doses (Fig. 2). Increasing doses of the compound produce graded decreases in all three hormones 24 h after a single oral administration.

To evaluate the mechanism underlying the reduction in plasma TSH, pituitary TSH β mRNA and TSH protein levels were examined 2 h after animals received a single administration of either vehicle or LG100268 (30 mg/kg). Although we showed that circulating TSH levels were significantly suppressed at this time point, there was no change in either TSH β mRNA or TSH protein levels in pituitaries from LG100268-treated animals (Fig. 3). These results indicate that the LG100268-induced acute hypothyroidism demonstrated above can not be explained by suppression of TSH β transcription or depletion of TSH stores.

To determine whether pituitary TSH secretion is affected by acute RXR agonist treatment, we studied TRH-stimulated TSH secretion in rats after a single oral administration of

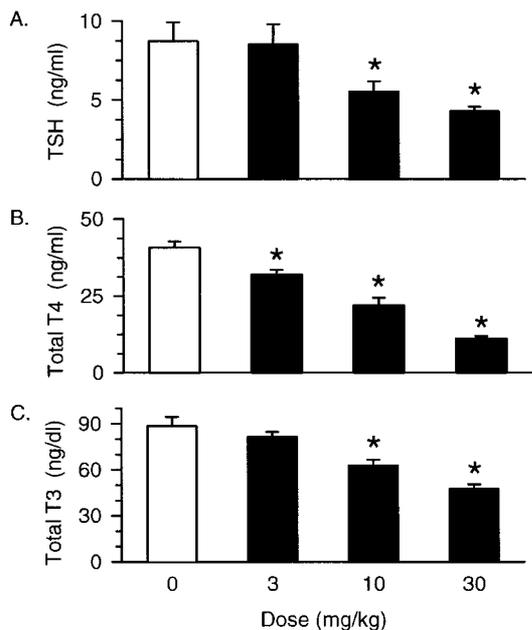


FIG. 2. LG100268 treatment dose-dependently reduces thyroid axis hormones. Male Sprague Dawley rats were treated orally with vehicle with or without LG100268 (3, 10, and 30 mg/kg). Blood was collected via decapitation 24 h after treatment, and serum was prepared and kept frozen at -20°C for RIA. Data are expressed as the mean and SEM ($n = 7$). Data were analyzed using one-way ANOVA (Dunnett's method). *, $P < 0.05$ vs. vehicle (0 mg/kg). A, TSH; B, total T₄; C, total T₃.

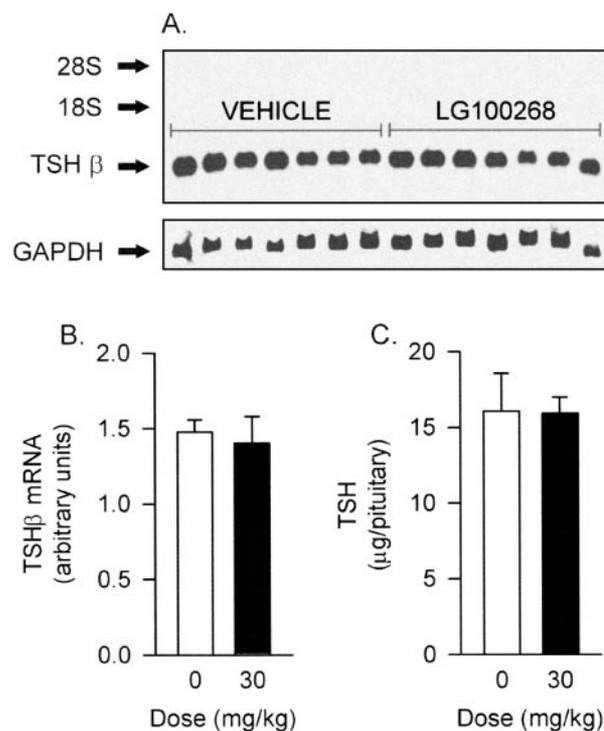


FIG. 3. Acute LG100268 treatment does not reduce pituitary TSH β mRNA or TSH content. Animals received either vehicle or LG100268 (30 mg/kg) 2 h before they were killed. Pituitaries were dissected and snap-frozen in liquid N₂, then stored at -80°C for mRNA or protein preparations. A, Northern blot of pituitary TSH β as well as GAPDH mRNA. B, TSH β mRNA levels (arbitrary units, normalized to GAPDH) were expressed as the mean \pm SEM ($n = 7$). C, Pituitary TSH content. TSH was analyzed using RIA in pituitary protein extracts. Data are expressed as the mean \pm SEM ($n = 7$).

either vehicle or LG100268 (30 mg/kg). Two hours after treatment, basal plasma TSH levels in LG100268-treated animals were significantly lower than those in vehicle-treated rats (3.95 ± 0.21 and 8.27 ± 0.81 ng/ml, respectively; mean \pm SEM; $P < 0.05$; Fig. 4A). TSH levels were increased by iv TRH ($3 \mu\text{g/kg}$) injection in both vehicle- and LG100268-treated animals. Maximum responses were observed 15 min after TRH injection (50.2 ± 3.31 and 21.9 ± 1.71 ng/ml in vehicle- and LG100268-treated animals, respectively). Injection of saline did not alter TSH levels in either vehicle- or LG100268-treated animals. To compare the TRH-stimulated increase in TSH levels over the 60-min period, incremental TSH AUCs were calculated for individual animals. LG100268 treatment reduced the AUC of TRH-stimulated TSH release from 1287 ± 93 min \times ng/ml for vehicle-treated animals to 592 ± 68 min \times ng/ml ($P < 0.05$; Fig. 4B), a decrease of 54%. These data suggest that LG100268 treatment reduces TSH secretion at the level of the rat pituitary. For individual animals there was a good correlation between basal and peak TSH levels (Fig. 4C; $r^2 = 0.63$; $P < 0.05$), although the data appear to cluster into two groups (treated and untreated).

Discussion

In the present study we have shown that LG100268, a potent and selective RXR agonist, rapidly reduced plasma TSH levels in rats. The reduction in circulating TSH levels is

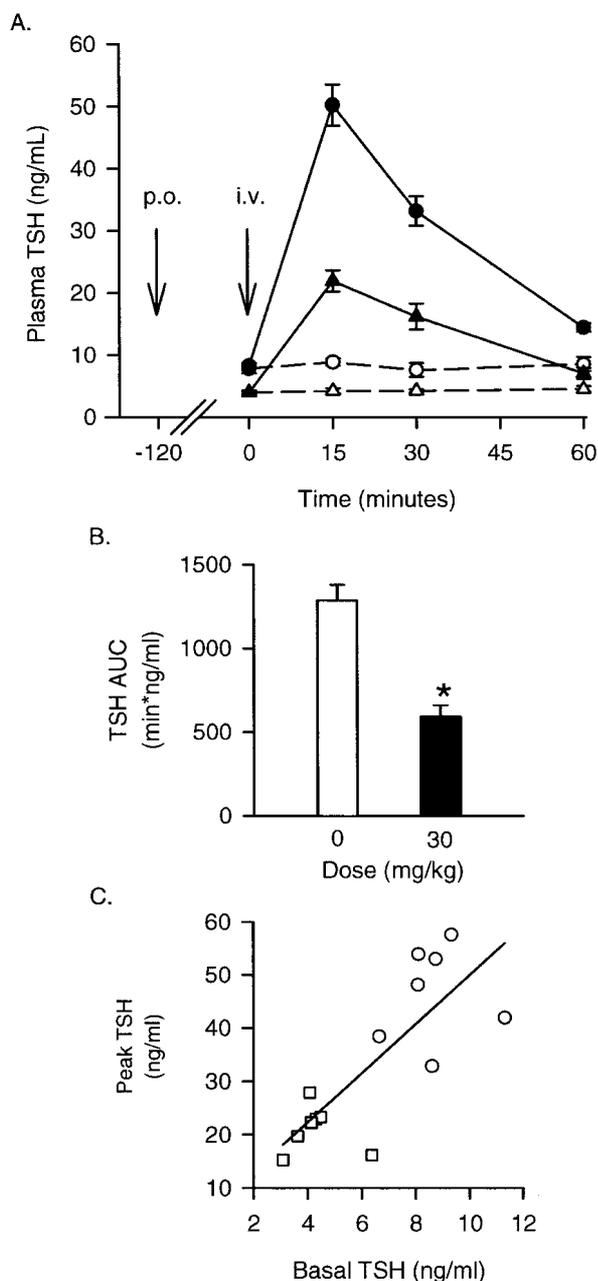


FIG. 4. LG100268 treatment blunts TRH-stimulated TSH secretion. Animals received a single oral administration (p.o.) of either vehicle or LG100268 (30 mg/kg) at -120 min. Two hours later, blood samples were collected via indwelling iv cannulas before (time zero) and 15, 30, and 60 min after an iv injection of either saline or TRH in saline (3 μ g/kg). A, Plasma TSH levels before and after iv injection of either saline or TRH. Data are expressed as the mean \pm SEM. \circ , Vehicle p.o. and saline iv; \bullet , vehicle p.o. and TRH iv; \triangle , LG100268 p.o. and saline iv; \blacktriangle , LG100268 p.o. and TRH iv. B, Incremental AUC (AUC above time zero TSH level) calculated on an individual animal basis for TRH-treated groups. Data are expressed as the mean \pm SEM. Statistical analysis was performed by *t* test. *, $P < 0.05$ vs. vehicle ($n = 7$ for both groups). C, Correlation analysis of basal and peak TSH levels for vehicle- and LG100268-treated rats. There is a significant correlation between basal and peak TSH levels ($r^2 = 0.63$; $P < 0.05$). \bullet and \circ , TSH levels in vehicle-treated rats; \blacktriangle and \triangle , TSH levels in LG100268-treated rats.

not caused by a decrease in pituitary TSH β mRNA or TSH protein. Rather, TRH-stimulated TSH secretion is blunted in LG100268-treated animals, indicating an impaired TSH secretory response from the pituitary.

It is known that retinoids suppress thyroid axis hormones in both humans and rodents. In humans, 9-*cis*-retinoic acid (9-*cis*-RA) treatment is associated with decreased TSH, T₄, and T₃ levels, and hormone levels return to normal within days after withdrawal of the treatment (14). In rats it has been shown that all-*trans*-retinoic acid (all-*trans*-RA) treatment decreased basal TSH levels and impaired TRH-stimulated TSH secretion (15). However, it is unclear whether it is the activation of RAR, RXR, or both that is responsible for the thyroid suppression. For example, 9-*cis*-RA can bind and *trans*-activate both RAR and RXR, and all-*trans*-RA can readily isomerize to 9-*cis*-RA (16, 17). Therefore, the role of RAR and/or RXR in the retinoid-induced suppression of thyroid axis hormones in those studies remains to be clarified.

Highly selective RXR agonists can be used to determine the relationship between RXR activation and thyroid function. LG100268 is such a highly selective agonist for RXR (6). It selectively binds to the RXR with high affinity (K_d 3 nM), whereas its binding affinity for RAR is low ($K_d > 10,000$ nM). In cotransfection assays, its 50% effective concentrations are 3–4 nM for RXRs and more than 10,000 nM for RARs (6). In the present study we have shown that LG100268 suppresses thyroid axis hormones in the rat, clearly associating suppression of the thyroid axis with RXR activation. Together with the observation that RXR γ -null mice have elevated levels of TSH and T₄ (18), these results indicate that RXR may play an active role in regulation of the thyroid hormone axis.

The mechanism(s) by which RXR suppresses circulating TSH levels *in vivo* is not completely understood. There are several lines of evidence indicating that TSH β gene expression might be the direct target. TSH β mRNA levels are increased in the pituitaries of vitamin A-deficient rats, and the elevated TSH β mRNA levels rapidly returned to normal after all-*trans*-RA treatment (19). It has also been shown that after 16 h of treatment with either 9-*cis*-RA or LGD346, an RXR agonist with a similar *in vitro* profile to LG100268, TSH promoter activity was significantly decreased (12). In addition, it has been demonstrated that 9-*cis*-RA suppresses the activity of the TSH β gene promoter *in vitro* through RXR γ 1 (20). Therefore, RXR-mediated suppression of TSH β gene expression could contribute to the mechanism for retinoid-induced hypothyroidism. However, in this study we demonstrated that there is a TSH β gene expression-independent mechanism involved in the acute LG100268-mediated suppression of circulating TSH levels in the rat. Plasma TSH levels decrease rapidly after LG100268 treatment. The decrease is apparent as early 0.5 h after oral administration, and becomes significant 2 h after treatment. It is unlikely that this rapid decline in circulating TSH levels is due to a reduction in TSH β mRNA, because we demonstrate that pituitary TSH β mRNA levels are not different between vehicle- and LG100268-treated animals 2 h after treatment, and the normal half-life for rat TSH β mRNA is more than 24 h (21). Thus, the acute reduction in circulating TSH levels that we observed after LG100268 administration is not due to an alteration in pituitary TSH β mRNA levels.

The apparent discrepancies between the unchanged mRNA levels observed in this study and the suppression of TSH β promoter activity by RXR ligands is probably due to the difference in the length of the treatment. We determined TSH β mRNA levels 2 h after treatment. In comparison, TSH promoter activities were measured 16 h after treatment with RXR ligands in the study mentioned above (12). Indeed, we have found that TSH β mRNA levels are decreased in Sprague Dawley rats after 10 d of treatment with LG100268 (unpublished observations). Therefore, the rapid effect that we have described represents an additional and unexpected mechanism that contributes to the acute LG100268-induced suppression of the thyroid axis.

If suppression of TSH β gene transcription is not responsible for the acute LG100268-induced decrease in circulating TSH levels that we have observed, then what is responsible? Our data point to a defect in the regulation of pituitary TSH secretion, rather than alteration in pituitary TSH β gene expression or the TSH storage pool. This is demonstrated by the observation that TRH-stimulated TSH secretion was blunted in rats treated with LG100268 without alterations in the level of either TSH β mRNA or TSH protein. The exact site of the defect in TSH secretion is unclear. It might lie at the TRH receptor, TRH receptor-induced signal transduction, or within the hormone secretory machinery. Further studies are needed to elucidate the molecular target for the RXR agonist-induced acute decrease in TSH secretion in the rat. Furthermore, it cannot be ruled out that there may be additional retinoid-associated thyroid axis defects at the hypothalamic level.

Previous investigators have demonstrated a significant correlation between basal and peak levels of TSH in humans (22, 23). We also observe such a correlation, although our data appear to fall into two clusters, representing treated and control animals. Spencer *et al.* (22) suggest that hypothyroidism in patients could be the result of either a reduction in TSH stores or a reduction in TRH receptor number. We have demonstrated that in Sprague Dawley rats neither the decline in basal nor that in TRH-stimulated TSH results from a reduction in TSH stores. A reduction in TRH receptor number could be responsible for the effects observed both in hypothyroid patients and in our experiments.

Treatment with RXR agonists is associated with two undesirable metabolic effects, suppression of thyroid axis hormones and elevation of triglycerides. Interestingly, there are several similarities between the LG100268-induced hypothyroidism and hypertriglyceridemia. First, both effects can be observed rapidly after treating naive animals, changing within hours after compound administration. These results are in sharp contrast to the LG100268-induced insulin sensitization in insulin-resistant/diabetic animal models, which takes days of treatment before becoming significant (9). Second, both activities appear to be the result of alterations in secretion. In the present study we show that the decrease in TSH appears to result from reduced TSH secretion, without a reduction of TSH β mRNA. The LG100268-induced elevation of triglycerides *in vivo* is thought to result from a reduction in lipoprotein lipase (LPL) activity in cardiac and skeletal muscle. This reduction in LPL activity was shown to occur without alteration in LPL mRNA and was believed to

result from a defect in secretion (24). Third, for both tissues affected, *i.e.* anterior pituitary and skeletal muscle, there is abundant RXR γ expression (20, 25). As RXR γ has a relatively limited tissue distribution, this suggests that both retinoid-induced suppression of the thyroid axis and the elevation of triglycerides might be mediated through RXR γ . Indeed, it has been shown that the RXR γ -null mice have elevated serum TSH levels (18), indicating an inhibitory role for RXR γ in the regulation of thyroid axis hormones. Further study with selective RXR agonists in RXR γ -null mice may help to determine the role of RXR γ in the retinoid-induced suppression of the thyroid axis and the elevation of triglycerides.

In summary, the acute reduction in circulating TSH levels induced by LG100268 treatment is not associated with changes in pituitary TSH β mRNA levels or TSH protein content. Our data demonstrate that LG100268 can apparently reduce pituitary TSH secretion through a mechanism independent of the regulation of TSH β gene transcription. Further work is required to determine the precise molecular mechanism of this activity.

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