RXR receptor agonist suppression of thyroid function: central effects in the absence of thyroid hormone receptor

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Macchia, Paolo E., Ping Jiang, Yan-Dar Yuan, Roslantha A. S. Chandarardna, Roy E. Weiss, Olivier Chassande, Jacques Samarut, Samuel Refetoff, and Charles F. Burant. RXR receptor agonist suppression of thyroid function: central effects in the absence of thyroid hormone receptor. Am J Physiol Endocrinol Metab 283: E326–E331, 2002; 10.1152/ajpendo.00313.2001.—High-affinity agonists for the retinoic acid X receptors (RXR) have pleotropic effects when administered to humans. These include induction of hypertriglyceridemia and hypothyroidism. We determined the effect of a novel high-affinity RXR agonist with potent antihyperglycemic effects on thyroid function of female Zucker diabetic rats and nondiabetic littermates and in db/db mice. In both nondiabetic and ZFF rats, AGN194204 causes a 70–80% decrease in thyrotropin (TSH), 3,3',5-triiodothyronine, and thyroxine (T₄) concentrations. In the db/db mouse, AGN194204 causes a time-dependent decrease in thyroid hormone levels with the fall in TSH that was significant after 1 day of treatment preceding the fall in T₄ levels that was significant at 3 days of treatment. Treatment with AGN194204 caused an initial increase in hepatic 5'-deiodinase mRNA levels which then fell to undetectable levels by 3 days of treatment and continued to be low at 7 days of treatment. After treatment for 5 days with AGN194204, both wild-type and thyroid hormone receptor β (TRβ⁻⁻)−deficient mice demonstrated a nearly 50% decrease in serum TSH and T₄ concentrations. The results suggest that a high-affinity RXR agonist with antihyperglycemic activity can cause central hypothyroidism in TRβ, the main mediator of hormone-induced TSH suppression.

nuclear receptors; retinoic acid receptors; hypothyroidism; knockout mice

THE LEVEL OF circulating thyroid hormones (TH), 3,3',5-triiodothyronine (T₃) and thyroxine (T₄), is tightly regulated by thyrotropin (TSH) in serum through a negative feedback mainly at the level of the pituitary thyrotrophs. It involves the binding of T₃ to specific nuclear TH receptors (TR) that interact with upstream specific DNA sequences located in the α- and β-subunits of TSH (29). The two TRs, α and β, are members of the nuclear receptor superfamily, which includes the vitamin D receptor, glucocorticoid and sex hormone receptors, retinoic acid (RAR) and retinoid X receptors (RXR), and the peroxisome proliferator-activated receptor (PPAR) isoforms (7, 19, 21). TRs act as ligand-dependent nuclear transcription factors that mediate the action of TH by positive and negative regulation of TH-responsive genes through binding to hormone response element (HRE) which consist of specific DNA sequences usually located in their promoter region (21). On the other hand, the cognate ligands of RAR and RXR, retinoic acid (RA) and 9-cis-retinoic acid (9-cis-RA), respectively, can transactivate through the same synthetic palindromic HRE and several natural HREs that are normally involved in TR-mediated gene regulation (32, 37). Specificity of gene transcription via RXR is thought to be mediated by the heterodimeric partner. Thus the RAR/RXR heterodimer binds to sequences oriented as direct repeats (DRs) that are separated by two or five nucleotides (DR-2 and DR-5). In contrast, PPAR/RXR heterodimers are thought to act via DR-1 elements, and TR/RXR can form complexes with DR-4 or DR-5 (6, 18, 20).

The RXR agonist Targretin has been used in clinical trials for cutaneous T-cell lymphomas (28). All patients treated for T-cell lymphoma with this RXR agonist developed reversible central hypothyroidism (28). Because TR-mediated transactivation induced by T₃ is facilitated by the formation of TR/RXR heterodimers (1, 24), the above finding suggested the possibility that...
9-cis-RA agonists may suppress TSH gene expression through binding to TR/RXR heterodimers. In contrast, in vitro studies suggest that the downregulation of TSH by 9-cis-RA is mediated through an RXR-dependent mechanism involving a region in the TSH promoter distinct from the one that mediates the T₃-dependent TSH suppression (14). However, this system provides for some degree of functional overspill. The latter has been observed in vitro but also in vivo. In the rat, vitamin A deficiency produces an increase in serum TSH that results in an increase in plasma THs, T₄ and T₃ (22), whereas excess vitamin A or RA has the opposite effect (5, 27). More recently, Brown et al. (3) showed that mice deficient in the RXRγ exhibited a mild resistance to TH. These effects appear to be mediated through the regulation of TSH gene expression (2).

High-affinity RXR agonists are under development as agents to treat insulin-resistant diabetes (31). One proposed mechanism of action is transactivation through a PPARγ/RXR heterodimer. In this model, similar to TR, PPARγ directs the heterodimeric complexes to distinct HRE sites, a DR-1, and the binding of ligand to RXR attracts cofactors to the complex that activates or suppresses PPARγ responsive genes in a manner similar to PPARγ ligands (23).

In the present study we examined the effect of novel RXR-ligand agonists on thyroid function in diabetic rodents and in a mouse deficient in TRβ. While of high affinity and selectivity for RXR, this agonist binds all three RXR isoforms. Our data indicate that the high-affinity RXR agonist can cause secondary hypothyroidism. To gain insight into the mechanism involved in the RXR ligand-mediated regulation of TSH in vivo, we made use of a knockout mouse (TRβ⁻/⁻) that does not express TRβ, the principal isoform that mediates TH-induced TSH suppression (8, 34). These mice have resistance to TH, and their responses to T₄ and T₃ have been characterized over a wide range of TH deprivation and replacement doses (34). The findings in TRβ-null mice demonstrate that the presence of TRβ is not required for downregulation of TSH by RXR ligands.

**MATERIALS AND METHODS**

**Animal Procedures**

All animal studies were approved by the Institutional Animal Care and Use Committee of Pfizer Global Research and Development-Ann Arbor Laboratories and the Institutional Animal Care and Use Committee at the University of Chicago.

*Rat studies.* Female Wistar and female Zucker diabetic fatty rats (ZDF/Gmi-fa/fa; ZDF rats) were purchased from Genetic Models (Indianapolis, IN) at 5–6 wk of age and were fed a semipurified high-fat diet (48% fat, 16% protein, diet no. 13004) prepared by Research Diets (New Brunswick, NJ). After the animals became hyperglycemic (fed blood glucose >250, ~3 wk on the diet), they were treated orally with vehicle (carboxymethyl cellulose) or AGN194204 (0.3 or 10 mg/kg) (14, 33) at 10:00 AM for 7 days. On the morning of the 8th day, the animals were anesthetized, and blood was collected by cardiac puncture.

*Mouse studies.* Male diabetic db/db (10-wk-old) mice were maintained on standard laboratory food treated by oral gavage with vehicle or AGN194204 at 4 mg/kg at 10:00 AM for 1, 3, or 7 days. They were killed after a 4-h fast when serum was obtained for TH measurements.

The method for the generation of TRβ-deficient (TRβ⁻/⁻) mice has been described elsewhere (10). These mice completely lack both TR isoforms (β1 and β2) encoded by the TRβ gene and are resistant to TH. They manifest the same features as those described in mice produced earlier by Forrest and co-workers (8, 9) and Weiss and co-workers (34, 35), including deafness (11). In all the experiments, wild-type (WT) mice were obtained by repeated crossings of heterozygous TRβ⁻/⁺ animals. Male mice were weaned on the fourth week after birth and were fed rodent diet containing 0.8 ppm iodine (no. 5053 lab diet) and given tap water ad libitum. They were housed, three to five mice per cage, in an environment controlled for temperature of 19°C and 12:12-h dark-artificial light cycles.

All mice were males, 60–70 days old at the beginning of the experiment. The weights of TRβ⁻/⁻ (23.5 ± 0.4 (SE) g) and WT (23.4 ± 0.5 (SE) g) mice were similar. Before starting the experiment, ~300 μl of blood were obtained from the tail vein under light methoxyflurane (Pitman Moore, Mundelein, IL) anesthesia (baseline). AGN194204 was diluted in phosphate-buffered saline (PBS) containing 5% DMSO to a final concentration of 0.5 mg/ml. Addition of AGN194204, dissolved in DMSO, to PBS produces partial precipitation of the compound. The suspension was thus carefully mixed before it was used for injection.

Seven mice of each genotype were injected intraperitoneally for five consecutive days with 4 mg AGN194204/kg body wt. A control group of the same size was treated with the vehicle alone (5% DMSO in PBS). Twelve to 16 h after the fifth injection, experiments were terminated by exsanguinations through retro-orbital vein puncture under methoxyflurane (Pitman Moore) anesthesia. Serum was separated by centrifugation and stored at −20°C until analysis.

**Measurements in Serum Samples**

Rat TSH was measured in 50 μl of serum using the rat TSH radioimmunoassay system form Amersham Pharmacia Biotech (Piscataway, NJ), according to the manufacturer’s instruction with minor modifications. Mouse TSH was measured in 50 μl of serum using a sensitive, heterologous, disulfuric acid, double-antibody precipitation radioimmunoassay as described (26). Results were expressed in terms of bioassayable TSH units, calibrated with bovine TSH (Sigma Chemical, St. Louis, MO).

Serum total T₄ and total T₃ concentrations were measured by radioimmunoassays using antibody-coated tubes (Diagnostic Products, Los Angeles, CA) in 25 and 50 μl of serum, respectively. The sensitivity of the assays was 0.2 μg T₄/dl (2.6 nM) and 20 ng T₃/dl (0.5 nM). The serum-free T₄ concentration was estimated from the free T₄ index, calculated as the product of the serum T₄ concentration and the T₄-resin uptake value. Whole blood glucose was measured using an HemoCue (Angelholm, Sweden). Triglyceride levels were determined with a commercial kit (Triglycerides/GB; Boeringer Mannheim, Indianapolis, IN).

**Northern Blotting**

Total RNA was extracted from liver of db/db mice at the time of death using TRIzol reagent. RNA was subjected to Northern analysis, hybridized overnight at 42°C with cDNA encoding mouse 5'-deiodinase (15), and labeled with
**RESULTS**

The high-affinity ligand for the RXR/RXR homodimer AGN194204 (33) was administered by gavage to Wistar rats (10 mg·kg⁻¹·day⁻¹) or ZDF rats (0.3 or 10 mg·kg⁻¹·day⁻¹) for 7 days. The diabetic rats showed a reduction in blood glucose to normal levels and an increase in serum triglyceride levels at both doses of the RXR activator, whereas the WT animals remained normoglycemic. There were no significant changes in weight in the RXR ligand-treated group (Table 1).

Evaluation of thyroid function tests in these animals showed that there was a significant decrease of serum total T₃ (TT₃) in both control and diabetic rats (Fig. 1B) after AGN194204 treatment. The serum total T₃ (TT₃) concentration was significantly decreased only in rats given 10 mg·kg⁻¹·day⁻¹ of the RXR agonist, although there was a trend to lower levels in the ZDF rats given the 0.3-mg/kg dose (Fig. 1C). The decline in TH levels was due to a central effect, either on the pituitary or hypothalamus, since there was a significant concomitant decrease in serum TSH levels (Fig. 1A). A dose-dependent decline in the free T₄ index, in parallel with that of TT₄, was also documented. Similar effects were observed in ZDF rats treated with a second high-affinity RXR-ligand, AGN195203 (data not shown). The decline in thyroid function was not strain specific, since Fisher rats, receiving continuous infusion of AGN194204 for 2 wk, similarly showed a decline in TT₄ and TT₃ and TSH levels (data not shown).

To ascertain the time course of changes in thyroid function by RXR agonists, db/db mice were treated for 1, 3, or 7 days with 4 mg/kg AGN194204, after which serum TH levels were measured. There was a significant decrease in serum TSH levels after 24 h of treatment, and these remained suppressed in animals treated for 3 and 7 days (Fig. 2A). The mean serum TT₄ levels fell significantly only on the third and seventh day of treatment (Fig. 2B), suggesting it was secondary to the fall in TSH. The decrease of thyroid hormone action in a peripheral tissue (liver) was demonstrated by the decline in the levels of mRNA encoding 5'-deiodinase (Fig. 2C). The reason for the small rise after 24 h of treatment is not readily apparent but may be due to a direct effect of the RXR agonist.

**Effect of the RXR Ligand Agonists in WT Mice and in Mice Deficient in TRβ**

To determine if the suppression of TSH by RXR agonists occurs through RXR/RXR homodimers rather than through the inhibiting effect of a liganded RXR/TR heterodimer, AGN194204 was given to WT

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**Table 1. Weight, serum glucose, and serum triglyceride data**

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Wild Type + AGN194204 (10 mg/kg)</th>
<th>Diabetic</th>
<th>Diabetic + AGN194204 (0.3 mg/ml)</th>
<th>Diabetic + AGN194204 (10 mg/kg)</th>
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</thead>
<tbody>
<tr>
<td><strong>Weight, g</strong></td>
<td>278 ± 12</td>
<td>273 ± 16</td>
<td>323 ± 4</td>
<td>353 ± 4</td>
<td>343 ± 5</td>
</tr>
<tr>
<td><strong>Serum glucose, mg/dl</strong></td>
<td>135 ± 12</td>
<td>129 ± 8</td>
<td>244 ± 53</td>
<td>135 ± 4</td>
<td>140 ± 16</td>
</tr>
<tr>
<td><strong>Serum triglyceride, mg/dl</strong></td>
<td>67 ± 7</td>
<td>161 ± 36</td>
<td>517 ± 52</td>
<td>2,902 ± 428</td>
<td>3,009 ± 388</td>
</tr>
</tbody>
</table>

Values are means ± SE.
mice and to TRβ−/− mice of the same strain that are deficient in TRβ (Fig. 3). WT mice treated with the vehicle showed random changes in serum TT4 and TSH levels with paired mean values not significantly different (P > 0.05). WT mice given 4 mg·kg body wt−1·day−1 AGN194204 for 5 days demonstrated a significant decrease in T4 and TSH levels (Fig. 3, A and C). Similar results were obtained in TRβ−/− mice (Fig. 3, B and D). The magnitude of serum TSH reduction after treatment with AGN194204 was not significantly (P = 0.055) different between TRβ−/− and WT mice (34.5 ± 10.8 and 70.0% ± 12.2% below baseline, respectively) because of wide scatter of values, whereas the decrease of serum T4 concentration was significantly (P < 0.001) greater in TRβ−/− compared with WT mice (39.7 ± 4.9 and 16.5 ± 2.2% below baseline, respectively). This is probably due to the higher serum TT4 levels in TRβ−/− mice at baseline.

DISCUSSION

The mechanism by which nuclear receptors control transcription of negatively regulated genes is not well understood. Although HREs have been identified in the proximal promoter region of genes negatively regulated by TH, their nature and structural requirement for ligand-dependent repression have not been defined (4, 17). Recent investigations have suggested two mechanisms for the transcriptional control of genes that are negatively regulated by the liganded TR. One proposes the partitioning of histone deacetylases and histone acetyltransferases between TR and other transcription factors that bind to the TSH subunits in promoters (30). Another suggests that TRβ2, through its unique AF-1 domain, specifically mediates a ligand-independent recruitment of coactivator to the transcription complex (25). The demonstration of ligand-specific RXR repression of TSH gene expression could
help identify additional mechanisms for transcriptional control.

Although Brown et al. (3) showed that T3 and 9-cis-RA acted independently on distinct sites on the TSHβ promoter to suppress gene expression, it was still unclear whether the 9-cis-RA required TR. To address this we tested the effect of a new RXR ligand agonist, AGN194204, in diabetic animals and showed that this compound induces a potent and rapid reduction in TH levels. The time course demonstrates that this is likely due to a rapid suppression of TSH levels (Fig. 2), similar to that seen in patients treated with Targretin for lymphoma (28). When we determined the effect of AGN194204 on the regulation of thyroid function in mice lacking the two products encoded by the TRβ gene (TRβ1 and TRβ2), the experiment yielded two findings. First, treatment with the RXR agonist reduced the serum TSH levels in the absence of the TRβ gene, indicating that in vivo the response to ligand-activated RXR is TRβ independent, very likely mediated by RXR without the recruitment of TR. Although we cannot exclude the involvement of TRα, this TR isofrom plays a lesser role in the downregulation of TSH (34). The second relevant finding is that the 9-cis-RA analog is equally potent in the suppression of TSH in WT and TRβ knockout mice, whereas TH has a lesser effect in these mice (34). The data thus suggest that this RXR agonist can override the resistance of thyrotrophs to TH.

Our results are in agreement with the in vitro studies suggesting a pathway for the 9-cis-RA-mediated suppression of TSH, independent of that of TR. Although the recent demonstration of TH resistance in RXR-deficient mice (3) does not exclude the role of TR through the formation of RXR/TR heterodimers, our results with TRβ-deficient animals indicate that TSH can be suppressed through RXR independently of TRβ in vivo. Although both T3 and 9-cis-RA could independently suppress TSHβ promoter activity, the combination of both ligands may produce a stronger suppressive effect than either alone (2), suggesting that these two hormones can cooperate in the TSHβ suppression. Thus RXR agonists may be useful in the suppression of TSH required for the control of thyroid cancer growth, especially in older individuals in whom administration of supraphysiological doses of TH are likely to produce cardiac complications. In addition, they could be useful in the treatment of patients with congenital resistance to TH, a condition in which the suppression of TSH cannot be accomplished readily with exogenous TH (36).

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