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Brokken, L.J.S.

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Suppression of Serum Thyrotropin by Graves' Immunoglobulins: Evidence for a Functional Pituitary Thyrotropin Receptor

Leon J.S. Brokken, Jolanda W.C. Scheenhart, Wilmar M. Wiersinga, and Mark F. Prummel

The Department of Endocrinology & Metabolism, Academic Medical Centre, University of Amsterdam, PO Box 22660, 1100 DD Amsterdam, The Netherlands

3.1 ABSTRACT

Antithyroid treatment for Graves’ hyperthyroidism restores euthyroidism clinically within 1-2 months, but it is well known that thyrotropin (TSH) levels can remain suppressed for many months despite normal free T₄ and T₃ levels. This has been attributed to a delayed recovery of the pituitary thyroid axis. However, we recently showed that the pituitary contains a TSH receptor (TSHR), through which TSH secretion may be downregulated via a paracrine feedback loop. In Graves’ disease, TSHR autoantibodies (TRAbs) may also bind this pituitary receptor, thus causing continued TSH suppression. This hypothesis was tested in a rat model. Rat thyroids were blocked by methimazole and the animals were supplemented with L-T₄. They were then injected with purified human IgG from Graves’ disease patients at two different titres or from a healthy control (TBII 591 U/L, 127 U/L and < 5 U/L). Despite similar T₄ and T₃ levels, TSH levels were indeed lower in the animals treated with high TRAb containing IgGs: 48-hour mean TSH concentrations (mean ± SEM; n = 8) were 11.6 ± 1.3 ng/mL as compared to 16.2 ± 0.9 ng/mL in the controls (P<0.01). The intermediate strength TRAb treated animals had levels in between the other two groups (13.5 ± 2.0 ng/mL). We conclude that TRAb can directly suppress TSH levels, independently of circulating thyroid hormone levels, suggesting a functioning pituitary TSH-receptor.
3.2 INTRODUCTION

Graves' disease is an autoimmune thyroid disorder characterised by circulating immunoglobulins directed against the thyrotropin receptor (TSHR) (1,2). The majority of these TSHR autoantibodies (TRAb) act as agonists by mimicking TSH binding leading to Graves' hyperthyroidism and goitre. Antithyroid drug treatment usually restores euthyroidism in 4-6 weeks in patients with hyperthyroidism (3). However, it may take much longer for thyrotropin (TSH) values to normalise. Many treated Graves' disease patients who are clinically euthyroid, and have normal T₄ and T₃ serum levels, continue to show decreased TSH levels (4,5).

The explanation for this continued suppression of TSH is unknown, but it is usually attributed to a delayed recovery of the pituitary-thyroid axis (6). We offer an alternative explanation, involving a direct effect of TRAb on TSH secretion by the pituitary. We have recently postulated that in addition to a negative feedback control by T₄ levels, TSH secretion is also influenced through a negative ultra-short feedback mechanism within the pituitary. We indeed demonstrated that the TSHR is expressed in the human anterior pituitary, on folliculostellate (FS) cells (7). When TSH is secreted by the thyrotrophs, it can bind to this receptor on FS cells, which then signal the thyrotrophs to decrease their TSH secretion. That the FS cells are involved in this feedback control is likely, because they are well known for their paracrine regulatory capabilities (8,9). Apart from this physiological control, the TSHR on FS cells may also bind circulating TRAb, which -by mimicking TSH- subsequently can cause a decrease in TSH secretion independently of thyroid hormone levels. Such a mechanism may very well be responsible for the observed low TSH levels in otherwise euthyroid Graves' patients under antithyroid drug treatment. For, TRAb often remain present in patients treated for Graves' disease (10,11), and can be responsible for the long time suppression of TSH.

To test this hypothesis, we used a modified "LATS-bioassay" (LATS = Long Acting Thyroid Stimulator) in which we measured the plasma TSH response to the administration of TRAb in rats that were unable to mount a thyroid response to TRAb by prior antithyroid drug treatment.
3.3 METHODS

Animals

Adult female Wistar rats (Harlan Sprague Dawley, Zeist, The Netherlands) weighing approximately 325 g were housed in cages at 21°C under a 12 h light/dark cycle, lights on at 7:00h and off at 19:00h. The animals received food and water ad libitum. The experiments described here were approved by the Animal Welfare Committee.

Experimental design

In order to suppress thyroidal T₄ production, 24 rats were treated with the antithyroid drug methimazole (1-methylimidazole-2-thiol, Sigma Chemicals, St. Louis, MO) at a concentration of 0.05% (w/v) in the drinking water, in combination with L-thyroxine (Sigma Chemicals, St. Louis, MO) dissolved in 0.9% NaCl (w/v) at 0.3 mg/mL and administered daily via a gastric tube in a dose of 1 mL/100 g body weight. These dosages were determined in pilot experiments and resulted in slightly elevated basal TSH levels between 5 and 10 ng/mL. After 1 week, at 9:00 am the animals received either a control IgG preparation (n=8), a TRAb-containing IgG preparation of intermediate strength (n=8) or a preparation with a high TRAb titre (n=8). Blood was collected in heparinised tubes just before, 1, 2, 4, 8, 24 and 48 h after administration of 1 mL of the appropriate IgG preparation and centrifuged at 3000 g for 10 min at 4°C. Plasma was stored at -20°C for later analysis. Administration of IgG and subsequent withdrawal of blood were performed via the tail vein under mild fentanyl/fluanisone/midazolam anaesthesia (0.25 mL per 100 g body weight). Hematocrite was determined before and 8 hours after the onset of the experiment.

Immunoglobulin purification

TRAb containing serum was obtained from 32 patients with Graves' disease who had TBII titres >100 U/L. Control serum was obtained from a healthy subject. The pooled TRAb containing serum and the control serum were filtered through a 0.22 μm low-protein binding filter (Millipore, Bedford, MA) and the IgGs were isolated by affinity chromatography as
described by Harlow and Lane (12). In short, the samples were passed over a 5 mL Protein G Sepharose column (HiTrap Protein G, Pharmacia Biotech) that was equilibrated with 0.1% BSA in run buffer (20 mM sodium phosphate buffer, pH 7.0). After washing with run buffer, the IgGs were eluted with 0.1 M glycine/HCl pH 2.7 and 1 mL fractions were collected in tubes containing 44 μL 1 M Tris pH 9.0 in order to neutralise the acid labile IgGs. The protein containing fractions were pooled and concentrated by ammonium sulphate precipitation (50%, w/v). The IgG preparations were dissolved in a minimal volume of phosphate buffered saline (PBS, pH 7.4) and finally dialysed for 16 h at 4°C against several changes of PBS. Both preparations were diluted in PBS to 30 mg/mL protein. TBI of the control IgG was < 5 U/L. The TRAb-containing IgG had a TBI titre of 591 U/L. To include an intermediate strength preparation, this high TRAb pool was partly diluted with control IgG yielding a TBI titre of 127 U/L. Purity of the IgG preparation and yield of the different IgG isotypes was assessed by immune electrophoresis and ELISA.

**Hormone assays**

TBI titres were measured by TRAK assay (Brahms Diagnostica, Berlin, Germany). TSH plasma levels were determined in a highly sensitive chemiluminescent enzyme immunoassay (Immulite Third Generation TSH kit, Rat TSH application, DPC, Los Angeles, CA). Total T\textsubscript{4} (TT\textsubscript{4}) and total T\textsubscript{3} plasma levels were determined by in-house radioimmunoassays (13) using rat null plasma as diluent. As an estimate of free T\textsubscript{4} levels, the free T\textsubscript{4}-index (FT\textsubscript{4}I) was calculated as the product of T\textsubscript{4} and T\textsubscript{3} resin uptake. The latter was determined with a T\textsubscript{3} Uptake Kit (Ortho-Clinical Diagnostics, Amersham, UK). Mean plasma levels of TSH, TT\textsubscript{4}, FT\textsubscript{4}I and T\textsubscript{3} levels over the 48-h period were calculated as the area under the curve divided by 48 h. All samples were measured within one run. Data are expressed as mean ± SEM.

**Statistical analysis**

The data was analysed using SPSS software, version 7.5.2 (SPSS Inc.). Time series were analysed by analysis of variance with repeated measurements and two grouping factors (time and treatment). Student’s t-test was used to compare the 48-h mean plasma levels. Differences between groups were considered significant at P < 0.05.
3.4 RESULTS

The pooled serum samples yielded IgG preparations that were > 99% pure with respect to total protein. Recovery of IgG₁, IgG₂ and IgG₃ was > 99%, and 85% for IgG₄.

At baseline, there were no differences in TSH, TT₄, T₃ and FT₄I between the three groups (Figure 3.1). After injection of IgG, thyroid function remained unaffected, as documented by similar T₃ levels in all three groups. TT₄ levels as well as FT₄I decreased transiently in all groups (Figure 3.1b, c, d). There were no statistically significant differences in TT₄, FT₄I and T₃ values between the three groups.

After injection of IgG, plasma TSH levels transiently increased in all three groups (Figure 3.1a). However, TSH levels in rats treated with high TRAb-containing IgG were lower during the whole observation period than in rats that received control IgG (P < 0.01 by ANOVA). Rats treated with intermediate strength TRAb showed TSH levels in between the other two groups.

The 48-h mean plasma hormone levels were calculated and showed no differences between the groups with respect to T₃, TT₄ or FT₄I (Figure 3.2). However, 48-h mean TSH plasma levels were significantly reduced in the rats treated with the highest concentration of TRAb (P < 0.01). Hematocrit did not change during the experiment (data not shown).

3.5 DISCUSSION

In this rat model we showed that TRAb are capable of suppressing TSH levels through an extrathyroidal pathway. Because, intravenous administration of TRAb containing human IgG, in contrast to normal control IgG, to methimazole-treated rats induced a decrease in TSH levels without affecting T₄ or T₃ levels. This extrathyroidal effect of TRAb is most likely caused by the binding of these IgGs to the TSHR in the pituitary. In a recent study, we have shown that the TSHR is expressed in the human anterior pituitary on the so-called folliculo-stellate (FS) cells (7). Others not only confirmed this finding (14), they also showed activation of adenylate cyclase by TSH in a mouse FS cell line. These cells make up ~10% of the pituitary cell population, and are known for their regulatory effects on pituitary hormone secretion (8,9). We hypothesised that these TSHR bearing FS cells play a role in fine-tuning of TSH secretion by the thyrotrophs through an ultra-short loop negative feedback mechanism.
Figure 3.1. **Left panel.** Rat plasma levels of TSH, TT$_4$, FT$_4$I and total T$_3$ during a 48-h period after the administration of 30 mg purified human IgG. (○) and (□) represent data after administration of TRAb containing IgG obtained from patients with Graves’ disease at intermediate and high concentrations, respectively. (●) represents data obtained after administration of control IgG. Curves of TRAb-treated animals are statistically compared to the control curves by ANOVA with repeated measurements and two grouping factors (time and treatment). NS, non significant, $P = 0.009$ denotes the difference between high TRAb serum versus control.

Figure 3.2. **Right panel.** Mean TSH, TT$_4$, FT$_4$I and T$_3$ plasma levels calculated over the 48-h time period in rats treated with control IgG (TBII < 5 U/L), intermediate strength TRAb (TBII 127 U/L), and high TRAb (TBII 591 U/L) containing IgG. ***, $P < 0.01$ compared to the control group.
possibly mediated via cytokines. In Graves' disease, this may have a further consequence. The anterior pituitary resides outside the blood-brain barrier and is thus accessible to circulating IgGs. So TRAb may very well bind to the TSHR on FS cells, which may then send a paracrine signal to the thyrotrophs to diminish their TSH secretion. The present rat study strongly supports this postulate.

We do not think that the observed suppression of TSH levels upon administration of TRAb-containing IgGs can be explained otherwise. First, we included a TBII negative, control IgG preparation that was administered in the same concentration as the two TBII positive preparations, thus correcting for aspecific general effects of IgGs on the pituitary-thyroidal axis. Next, we used two concentrations of TRAb-containing IgG and found a suggestion for a dose-response effect. Thirdly, the thyroid hormone levels were similar in all three groups over a 48-h period, and T4 and FT4 levels actually decreased slightly in all groups. This not only shows the effectiveness of the methimazole-induced block of thyroid hormone synthesis, but it also makes it highly unlikely that TRAb suppressed TSH levels via stimulation of the thyroid gland. In view of these considerations, we strongly believe that the TRAb sera indeed suppressed TSH levels via an extrathyroidal pathway.

We found that TSH levels increased rather sharply shortly after the administration of IgGs in all groups. We suggest that this was due to a stress response in the animals. Similar increases in TSH levels were found upon skin incision in patients undergoing a cholecystectomy (Endocrine Society, San Diego, 1999). In addition, part of the TSH increase may be explained by the naturally occurring morning surge in rats (15). We made our rats slightly hypothyroid in view of the mildly elevated TSH levels. This was done to ascertain that changes in TSH levels would indeed be detectable by the TSH assay.

We suggest that these data can be extrapolated to the human situation. When patients with Graves' hyperthyroidism are rendered euthyroid, it is frequently seen that their TSH levels remain suppressed for a long time, despite normal thyroid hormone levels (4,5). It is also known that TRAb can remain present for a variable period of months to even years and our data now support the hypothesis that TRAb may be responsible for extrathyroidal TSH suppression. We feel that this is a better explanation for continued TSH suppression than a delayed recovery of the pituitary-thyroid axis. The hypothesis can also explain another poorly understood phenomenon encountered in clinical practice. After one year of antithyroid drug treatment, approximately 50% of Graves' patients relapse. This occurs in patients with large goitres, in patients with high TBII titres (16,17), but also in those who continue to have
suppressed TSH levels in the absence of detectable TBII titres (18). We suggest, that these suppressed TSH values result from biologically active TRAb below the detection limit of routine TBII assays.

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3.7 REFERENCES


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