Thyrotropin-releasing hormone time-dependently influences thyrotropin microheterogeneity – an in vivo study in euthyroidism

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Abstract

Thyrotropin (TSH) is secreted not as one distinct hormone, but rather as a group of isohormones which differ in their oligosaccharide composition. Although the mechanisms regulating TSH glycosylation are not fully understood, there is strong evidence that TRH plays an important role. The aim of our study was to determine the dynamic influence of TRH on TSH microheterogeneity.

Sera were obtained from euthyroid volunteers (n=20) before and 30, 60, 120, 180 and 240 min after intravenous, nasal and oral administration of TRH in three independent runs (randomized order, at a time-interval of 3 weeks between each run). TSH was immuno-concentrated and analysed by isoelectric focusing (IEF) and lentil lectin affinity chromatography. TSH immunoreactivity was measured by an automated second-generation TSH immunoassay. Overall, serum TSH concentrations reached maximal values 30 min after intravenous, 60 min after nasal and 180 min after oral TRH stimulation. IEF analysis revealed 63·3±3·3% of pituitary standard TSH (IRP 80/558) in the neutral pH range (8>pH>6). In contrast, 30 min after TRH stimulation 80·8±3·7% (P<0·001) and 60 min after TRH stimulation 44·9±2·2% (P<0·001) of the TSH of euthyroid probands were found in this pH range, whereas 180 min after TRH stimulation 58·4±2·3% (P<0·001) were detected in the acidic pH range (pH<6). This shift of TSH composition in euthyroidism after TRH stimulation was confirmed by lentil lectin analysis of TSH: core-fucose content of euthyroid TSH was 73·4±3·8% 30 min and 22·9±3·2% 120 min after TRH stimulation in contrast to basal (53·3±1·8%; P<0·001) and pituitary standard (IRP 80/558) TSH (63·0±0·9%; P<0·001).

In conclusion, in euthyroidism, TRH stimulation time-dependently changes the distribution pattern of the TSH isoforms from an alkaline and neutral to a more acidic one. This corresponds to the secretion of isohormones with altered bioactivity which could influence the fine-tuning of thyroid function.

Journal of Endocrinology (2000) 166, 137–143

Introduction

Thyrotropin (TSH) is a glycoprotein consisting of an α-subunit, which is common among the members of the glycoprotein hormone family (TSH, luteinizing hormone, follicle-stimulating hormone and chorionicadotropin (CG)), and a hormone specific β-subunit (Keel 1989). The α-subunit contains two asparagine-linked oligosaccharides, whereas the β-subunit contains one (Pierce & Parsons 1981). Previous studies have revealed various TSH isoforms (isohormones), which differ in physical properties such as size and charge, as well as in biological activity and metabolic clearance rate (Thotakura et al. 1992, Schaaf et al. 1995, 1997). From very early on this heterogeneity had been attributed to the carbohydrate component of TSH (Webster et al. 1972, Baenzinger & Green 1988), and was documented by changes in the isoelectric points (Yora et al. 1979, Sergi et al. 1991, Robertson et al. 1997) as TSH isohormones with pH<6 possess a higher degree of negatively charged carbohydrate residues compared with TSH isoforms with pH>7 (Keel 1989).

The oligosaccharide composition is known to be modulated during TSH biosynthesis by various physiological endocrine factors (Mernez-Ferreira et al. 1986). One important factor has been shown to be thyrotropin-releasing hormone (TRH) (Weintraub et al. 1990). Although the influence of TRH on TSH glycosylation is well documented (Beck–Pecoz et al. 1985, Magnier 1990, 1994), there are no data available concerning a possible time-dependent influence of TRH on TSH microheterogeneity in vivo. We therefore performed isoelectric focusing (IEF) and lectin chromatography on circulating TSH
from euthyroid subjects after intravenous, nasal and oral TRH administration.

**Materials and Methods**

**Subjects**

Serum samples were obtained from euthyroid volunteers (n=20 males, aged 30-67 years, TSH 1-6 ± 0.8 μU/ml). Peripheral thyroid hormone parameters of free triiodothyronine (fT3) and free thyroxine (fT4) were within the normal ranges. To rule out morphological abnormalities, an ultrasound of the thyroid gland was performed using a 7.5 MHz linear transducer. Thyroid volume was 11-9 ± 4.3 ml. To ensure that pituitary function was normal, prolactin was measured before (11.8 ± 4.3 ng/ml) and 30 min after i.v. TRH stimulation (30.9 ± 12.9 ng/ml). Sera for TSH analysis were obtained before and 30, 60, 120, 180 and 240 min after TRH application at 0800 h after an overnight fast. TRH (Antepan; Henning, Berlin, Germany) was applied in a randomized order intravenously (200 μg), nasally (1 mg, one burst left nostril), or per os (40 mg tablet). There was an interval of 3 weeks between different modes of TRH application. Blood samples for analysis were centrifuged and the serum was frozen at -20°C.

Written informed consent was obtained from each person and the investigation was approved by the local ethical committee. In addition, human standard pituitary TSH (IRP 80/558; National Institute for Biological Standards and Controls, Potters Bar, Herts, UK) was applied. Bound material was eluted with 7 M column buffer, pH 7-4, containing in addition 0.5 M methyl α-D-mannopyranoside. Fractions (1 ml) were collected at a flow rate of 30 ml/h at room temperature. The recovery of TSH from the lectin–Sepharose 4B was approximately 90%. Further details of this method have been described previously (Miura et al. 1989).

**Isoelectric focusing**

A vertical polyacrylamide minigel system (Mighty Small II; Hoefer Scientific Instruments, Nuernberg, Germany) was utilized. For pI measurement, a pI marker protein kit from US Biochemicals (Cleveland, OH, USA) with pI values between 10-6 and 4-1 was employed. Separation of TSH isoforms was achieved within 3 h. Immediately at the end of the run the migration of the pI marker proteins was measured and each lane was cut into 2 mm slices. TSH was eluted overnight at room temperature in 1 ml Tris-buffer and measured by immunochromiluminescence assay. In duplicate runs, TSH isoform distribution patterns differed by less than 5% (for further details see Robertson et al. 1987). Typical isoelectrofocusing profiles are shown in Fig. 1.

**Immunometrical assays**

Serum levels of fT3, fT4 and prolactin were determined by standard assays. TSH in each sample was measured with an automated sandwich chemilumimometric assay (ACS TSH; Chiron Diagnostics, Fernwald, Germany), based on a combination of coupled polyclonal anti-hTSH antibody (sheep) and fluorescence-labeled monoclonal anti-hTSH antibody (mouse). The analytical sensitivity of this assay was 0.015 mIE/l and the cross-reactivity with human CG was less than 1%. To rule out the possibility that this assay does not recognize all TSH isoforms, TSH isoforms isolated by lectin affinity chromatography were diluted and measured with another highly sensitive TSH assay (CoTube TSH IRMA; BioRad, München, Germany; analytical sensitivity 0.02 mIE/l). This assay is based on a combination of coupled polyclonal anti-hTSH antibody (mouse) and 125I-labeled monoclonal anti-hTSH antibody (mouse). TSH values did not differ significantly in the two assays. For the ACS TSH assay, intra- and interassay precisions were 3-8 and 6-6% respectively.

**Statistical analysis**

For statistical analysis each run was divided into seven pH groups according to the protein marker kit as follows. pH range 1: 10 ≤ pH < 11; pH range 2: 9 ≤ pH < 10; pH range 3: 8 ≤ pH < 9; pH range 4: 7 ≤ pH < 8; pH range 5: 6 ≤ pH < 7; pH range 6: 5 ≤ pH < 6; pH range 7:
4 ≤ pH < 5. pH ranges 1–3 (≥ 8) comprise alkaline, pH ranges 4–5 (8 > pH ≥ 6) neutral and pH ranges 6–7 (pH < 6) acidic isoforms.

The percentage ± s.d. of TSH immunoreactivity and area under the curve found in each pH interval was calculated. S.E.M. was calculated as s.d. divided by the number of experimental runs. Statistical analysis was carried out with the unpaired, two-tailed Student’s t-test and appropriate analysis of variance. If not stated otherwise, levels of significance were established at P < 0.05.

Results

Total serum TSH concentration reached maximal values 30 min after intravenous, 60 min after nasal and 180 min after oral TRH stimulation (Fig. 2).

IEF analysis revealed 63.3 ± 3.3% of pituitary standard TSH (IRP 80/558) in the neutral pH range (8 > pH > 6). In contrast, 30 min after TRH stimulation 80.8 ± 3.7% (P < 0.001) and 60 min after TRH stimulation 44.9 ± 2.2% (P < 0.001) of the TSH of euthyroid probands were found in this pH range, whereas 180 min after TRH stimulation 58.4 ± 2.3% (P < 0.001) were detected in the acidic pH range (pH < 6) (Fig. 3).

Lentil lectin analysis of different TSH preparations revealed that the core-fucose content of euthyroid TSH was significantly increased 30 min and 120 min after TRH stimulation (e.g. to 73.4 ± 3.8% after nasal TRH stimulation in contrast to basal (53.3 ± 1.8%; P < 0.001) and pituitary standard (IRP 80/558) TSH (63.0 ± 0.9%; P < 0.001) (Fig. 4). A highly significant decrease in core-fucosylated TSH (P < 0.001) was found 120 min after TRH stimulation.

Whereas there was no significant difference in core-fucose content 30 min after TRH stimulation between the three different modes of TRH application, core-fucose content of TSH 120 min after oral stimulation was significantly diminished (P < 0.001) in comparison with
TSH 120 min after nasal and intravenous stimulation (Fig. 4).

Discussion

Releasing hormones such as gonadotropin–releasing hormone are known to induce not only changes in the quantity (greater numbers of molecules) but also in the quality (differences in glycosylation) of the secreted glycoprotein hormone by acting directly at translation and distal glycosylation levels (Ulloa-Aguirre et al. 1995, Perez & Apfelbaum 1996, Anobile et al. 1998, Lambert et al. 1998, Savastano et al. 1998, Timossi et al. 1998). As far as the pituitary–thyroid axis is concerned, the availability of an efficient immuno-affinity method for the purification and
concentration of serum TSH molecules (Schaaf et al. 1995, Persani et al. 1998) allows separation of different TSH isohormones. IEF and lectin affinity chromatography are common methods used to separate isoforms of glycoprotein hormones (Persani et al. 1998, Trojan et al. 1998). In our in vivo study in euthyroid probands we found a time-dependent shift of TSH composition after TRH stimulation from a neutral pH range to an acidic pH range 30 to 180 min after TRH stimulation. This shift of TSH composition was confirmed by lectin analysis. Core-fucose content significantly decreased after TRH stimulation in contrast to core-fucose content of basal euthyroid TSH and pituitary standard TSH.

TSH was measured with a highly sensitive immunoassay that does not discriminate between different TSH isoforms (Schaaf et al. 1995, Trojan et al. 1998). In accordance with the literature, we showed a maximal TSH concentration 30 min after intravenous, 60 min after nasal and 180 min after oral TRH stimulation (Schurr et al. 1985). Corresponding to previous results, IEF analysis of IRP hTSH revealed ≈ 25% alkaline, ≈ 53% neutral and ≈ 22% acidic TSH isoforms (Pickles et al. 1992, Magner 1994, Schaaf et al. 1997).

Different pH ranges of TSH isohormones are due to a variable content of sulfate and sialic acid residues (Baenzinger & Green 1988). TSH with different degrees of sialylation has been shown in various physiological and pathological states (Papandreou et al. 1993, Persani et al. 1998).

In humans and experimental animals, TSH secreted during hypothyroidism was found to be more sialylated than in euthyroid individuals (Miura et al. 1989, Pickles et al. 1992, Trojan et al. 1994). Further studies on the cellular mechanisms underlying this phenomenon have shown increased expression of α-2,6-sialyltransferase, β-1,4-galactosyltransferase, and the Golgi enzyme α-mannosidase-II in hypothyroid mice (Pickles et al. 1992, Helton & Magner 1994). As sialylation has an important effect on TSH metabolic clearance rate and bioactivity, it can be speculated that the increased percentage of the sialylated isoforms in hypothyroidism is part of a mechanism that provides an enhanced stimulation of the impaired thyroid function.

One possible mediator of changes in TSH glycosylation is known to be TRH. In euthyroid individuals, the i.v. administration of TRH releases TSH containing more core-fucose residues than basal TSH (Magner et al. 1992). In our present study we found a time-dependent increase in more acidic TSH isoforms.

Looking at core-fucose content of TRH-stimulated TSH, we found a significantly increased content 30 min after TRH stimulation, which was independent of the mode of TRH application, while 120 min after TRH application core-fucose content was significantly diminished. During the post-translational processing of TSH, oligosaccharide chains are added and modified. A quite late event in this pathway is the addition of galactose versus N-acetylgalactosamine. Oligosaccharide chains containing exposed galactose residues may be further modified by attaching terminal sialic acid, while N-acetylgalactosamine residues may become sulfated (Baenziger & Green 1988). In this context, the results of Trojan et al. (1998) show that patients with subclinical and overt primary hypothyroidism have a markedly increased proportion of serum TSH isoforms bearing terminal galactose and sialic acid residues. These results are based on additional analyses of

![Figure 4 Core-fucose content of euthyroid TSH before, 30 min and 120 min after TRH stimulation. Core-fucose content significantly increased (P<0.001) 30 min after TRH stimulation, and 120 min after TRH stimulation it significantly decreased.](http://www.endocrinology.org)
neuraminidase-treated TSH (Trojan et al. 1998), whereas our present study does not provide such data because of lack of material. Therefore, the change in pH of TRH-stimulated TSH can be attributed to increased sulfate and/or sialic acid content.

In a previous study, we showed that the two dominant intracellular signal transduction systems (cAMP formation and inositol phosphate release) are activated to different degrees by hTSH glycosylation variants (Schaaf et al. 1997). TSH isoforms separated by IEF showed significantly enhanced bioactivity for the basic isoforms and only low activity for the acidic forms. However, the attempt to establish a simple correlation between biochemical structure and biological activity of isoforms leads to controversial results (Pickles et al. 1992, Szkudlinski et al. 1993, Metcalfe et al. 1998, Persani et al. 1998). The less-processed basic isoforms such as desialylated, high-mannose, and core-fucosylated glycosylation variants have higher in vitro bioactivity. The degree of sialylation also seems to determine the in vivo potency of the glycoprotein hormone by influencing its metabolic rate and distribution (Szkudlinski et al. 1995).

On the other hand, extensive research concerning the response of TSH to TRH has revealed a blunted TSH maximum rise in depressed patients (Baumgartner 1993). The hypothesis was put forward that the decrease in TSH and prolactin response to TRH in depression is due to down-regulation of TSH receptors after TRH hypersecretion (Garbutt et al. 1994). Despite this change in thyrotropin function, most patients with depression are euthyroid. However, slightly elevated amounts of total and fT4, decreased amounts of total and fT3 and elevated amounts of reverse T3 have been reported (Baumgartner 1993). In view of an altered bioactivity by different isoform patterns a possible influence of TRH hypersecretion on the pituitary–thyroid axis in depression should be taken into account.

In summary, we have shown for the first time that TRH stimulation time-dependently changes TSH composition in euthyroidism. The distribution pattern of TSH isoforms changes from an alkaline and neutral to a more acidic TSH composition. This corresponds to the secretion of isoforms with altered bioactivity, thus possibly influencing the fine-tuning of thyroid function.

Acknowledgements

This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG Scha 701/1–1) and the Forum Schilddrüse e.V. We thank Dr A Bergmann (Brahms GmbH, Berlin, Germany) for supplying coated tubes and Dr Butz (Chiron Diagnostics GmbH) for supplying ACS TSH kits. We are indebted to Dr P Lohrer for correcting the English in this manuscript.

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Received 19 November 1999
Accepted 16 February 2000