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 $63.3 \pm 3.3\%$ of pituitary standard TSH (IRP 80/558) in the neutral pH range ($8 > \text{pH} > 6$). In

contrast, 30 min after TRH stimulation $80.8 \pm 3.7\%$ ($P < 0.001$) and 60 min after TRH stimulation $44.9 \pm 2.2\%$ ($P < 0.001$) of the TSH of euthyroid probands were found in this pH range, whereas 180 min after TRH stimulation $58.4 \pm 2.3\%$ ($P < 0.001$) were detected in the acidic pH range ($\text{pH} < 6$). 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 $73.4 \pm 3.8\%$ 30 min and $22.9 \pm 3.2\%$ 120 min after TRH stimulation in contrast to basal ($53.3 \pm 1.8\%$; $P < 0.001$) and pituitary standard (IRP 80/558) TSH ($63.0 \pm 0.9\%$; $P < 0.001$).

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.

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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 choriogonadotropin (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 $\text{pI} < 6$ possess a higher degree of negatively charged carbohydrate residues compared with TSH isoforms with $\text{pI} > 7$ (Keel 1989).

The oligosaccharide composition is known to be modulated during TSH biosynthesis by various physiological endocrine factors (Mernezes-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-Peccoz *et al.* 1985, Magner 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 30.6 ± 6.7 years, TSH 1.6 ± 0.8 $\mu\text{U}/\text{ml}$). 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 studied.

Immunoaffinity purification of human serum TSH

TSH from serum samples (10 ml) was purified with anti-human (h) TSH antibody-coated polystyrene tubes (SchAAF *et al.* 1995). In brief, 0.5 ml serum was incubated per tube at room temperature for 4 h. The supernatant was then decanted and antibody-bound TSH eluted with 0.2 M glycine-HCl (pH 2.2). Aliquots were diluted with 0.01% bovine serum albumin (BSA), pooled, and concentrated on the Centriprep ultrafiltration system (Amicon GmbH, Witten, Germany).

Lentil lectin affinity chromatography

Lectin columns containing 1 ml lentil lectin-Sepharose 4B (Pharmacia Biotech GmbH, Freiburg, Germany) were constructed in 10 ml glass pipettes. After loading 0.5 ml aliquots, equilibration was reached after 60 min. Unbound TSH was eluted with 12 ml column buffer, pH 8.0, containing 10 mM sodium chloride, 1 mM each of magnesium chloride, manganese chloride and calcium chloride, 0.1% sodium azide, and 0.1% BSA of analytical

grade (protease-free). Bound material was eluted with 7 ml 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 250; 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 immunochemiluminescence 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 chemiluminometric 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 ^{125}I -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: $10 \leq \text{pH} < 11$; pH range 2: $9 \leq \text{pH} < 10$; pH range 3: $8 \leq \text{pH} < 9$; pH range 4: $7 \leq \text{pH} < 8$; pH range 5: $6 \leq \text{pH} < 7$; pH range 6: $5 \leq \text{pH} < 6$; pH range 7:

Figure 1 Typical IEF analysis of (a) human standard pituitary TSH (IRP 80/558), and TSH levels in a euthyroid proband (b) 30 min, (c) 60 min and (d) 180 min after TRH stimulation.

$4 \leq \text{pH} < 5$. pH ranges 1–3 (≥ 8) comprise alkaline, pH ranges 4–5 ($8 > \text{pH} \geq 6$) neutral and pH ranges 6–7 ($\text{pH} < 6$) acidic isoforms.

The percentage $\pm$ 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 $63.3 \pm 3.3\%$ of pituitary standard TSH (IRP 80/558) in the neutral pH range ($8 > \text{pH} > 6$). In

contrast, 30 min after TRH stimulation $80.8 \pm 3.7\%$ ($P < 0.001$) and 60 min after TRH stimulation $44.9 \pm 2.2\%$ ($P < 0.001$) of the TSH of euthyroid probands were found in this pH range, whereas 180 min after TRH stimulation $58.4 \pm 2.3\%$ ($P < 0.001$) were detected in the acidic pH range ($\text{pH} < 6$) (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 $73.4 \pm 3.8\%$ after nasal TRH stimulation in contrast to basal $53.3 \pm 1.8\%$; $P < 0.001$) and pituitary standard (IRP 80/558) TSH ($63.0 \pm 0.9\%$; $P < 0.001$) (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

Figure 2 Serum concentration of total TSH $\pm$ s.d. after intravenous, nasal and oral TRH stimulation. Serum TSH concentration reached maximal values 30 min after intravenous, 60 min after nasal, and 180 min after oral TRH administration.

Figure 3 Percentage distribution $\pm$ s.d. of TSH isoforms separated by IEF in different pH ranges. Standard TSH (IRP 80/558) is compared with TRH-stimulated TSH of euthyroid probands ($n=20$) 30 min, 60 min and 180 min after TRH stimulation. Thirty minutes after intravenous TRH stimulation the highest percentage of TSH isoforms are found in the neutral pH range, while 60 min after nasal and 180 min after oral TRH stimulation there is an almost equal distribution of TSH isoforms in the neutral and acidic pH ranges, with a slight preference for acidic isoforms.

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

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.

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 lentil 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 $\approx 25\%$ alkaline, $\approx 53\%$ neutral and $\approx 22\%$ 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 (Baenziger & 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

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 isohormone 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 isohormones changes from an alkaline and neutral to a more acidic TSH composition. This corresponds to the secretion of isohormones with altered bioactivity, thus possibly influencing the fine-tuning of thyroid function.

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