Modulation of human thyrotropin oligosaccharide structures – enhanced proportion of sialylated and terminally galactosylated serum thyrotropin isoforms in subclinical and overt primary hypothyroidism

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Abstract

Enhanced sialylation of thyrotropin (TSH) prolongs its metabolic clearance rate and thus increases the hormone's *in vivo* bioactivity. This has been shown for hypothyroid rats and for recombinant human TSH, but there are few data on the sialylation of human serum TSH. The aim of this work was to further study sialylated human serum TSH, its precursors bearing terminal galactose residues, and the role of pharmacological doses of thyrotropin-releasing hormone (TRH) on their secretion under different degrees of primary hypothyroidism.

We analyzed serum TSH in patients with subclinical ($n=9$) and overt primary hypothyroidism ($n=13$) compared with euthyroid individuals ($n=12$) and human standard pituitary TSH (IRP 80/558). Blood was drawn before and 30 min after intravenous administration of 200 µg TRH, and TSH was purified by immunoaffinity concentration. The content of sialylated (sialo-) TSH and isoforms bearing terminal galactose (Gal-TSH, asialo-Gal-TSH) was measured by *Ricinus communis* (RCA 120) affinity chromatography in combination with enzymatic cleavage of sialic acid residues. TSH immunoreactivity was measured by an automated second generation TSH immunoassay.

Pituitary TSH contained $16.5 \pm 0.8\%$ Gal-TSH. In euthyroid individuals the proportion of Gal-TSH was

$14.6 \pm 1.9\%$, whereas TSH in patients with subclinical and overt primary hypothyroidism contained $23.9 \pm 3.5\%$ ($P<0.05$ vs euthyroid individuals) and $21.1 \pm 1.7\%$ Gal-TSH respectively. The mean ratio of asialo-Gal TSH was $23.8 \pm 0.6\%$ for pituitary TSH, $35.7 \pm 4.2\%$ in euthyroid individuals, $48.0 \pm 3.3\%$ in patients with subclinical, and $61.5 \pm 3.8\%$ ($P<0.001$ vs euthyroid individuals) in patients with overt primary hypothyroidism. For pituitary TSH the calculated proportion of sialo-TSH was $6.5 \pm 0.2\%$, for euthyroid individuals $20.3 \pm 2.8\%$, for patients with subclinical hypothyroidism $24.1 \pm 3.0\%$, and for patients with overt primary hypothyroidism $40.7 \pm 3.0\%$ ($P<0.001$ vs euthyroid individuals). The proportions of Gal-TSH, asialo-Gal-TSH, and sialo-TSH did not differ significantly before and after TRH administration in the individuals studied.

Our data 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, which may represent a mechanism for the further stimulation of thyroid function. Pharmacological doses of TRH cause an increased quantity of TSH to be released, but do not significantly alter the proportion of sialylated or terminally galactosylated TSH isoforms.

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Introduction

Thyrotropin (TSH) is a heterodimer glycoprotein hormone consisting of an α -subunit and a β -subunit. The α -subunit has two asparagine-linked oligosaccharide units, whereas the β -subunit has only one. Due to differential processing of oligosaccharides during TSH biosynthesis, these structures are heterogeneous: they differ in galactose, mannose and fucose content, branching properties and in

the degree of sulfation and sialylation. This microheterogeneity accounts for the fact that TSH is secreted not as a single structure but as a set of glycosylation variants. This has been demonstrated for animal TSH, human TSH (hTSH), and recombinant hTSH expressed in mammalian cell lines (Magner 1990, 1994).

The oligosaccharide residues of TSH are important for the association of subunits, secretion, stability, *in vitro* biological activity, and, most importantly as they influence

Table 1 Clinical data of euthyroid individuals and patients with subclinical and overt primary hypothyroidism. Data shown are means ± S.E.M. Normal ranges for TSH, total T₃, total T₄, and free T₄ are given in parentheses

	Euthyroid individuals	Subclinical hypothyroidism	Overt primary hypothyroidism
Number (female:male)	12 (9:3)	9 (7:2)	13 (10:3)
Age (years)	37 ± 4	32 ± 4	46 ± 1
Cause of hypothyroidism			
Chronic autoimmune thyroiditis*		5	6
Posttherapeutic ^a		2	5
Unknown		2	2
TSH (0.4–5.5 mIU/l)	11.8 ± 0.3	19.5 ± 4.2	54.4 ± 11.3
TRH-TSH ^b (mIU/l)	12.8 ± 1.5	79.4 ± 22.4	129.8 ± 23.8
Total T ₃ (0.7–2.0 ng/ml)	1.3 ± 0.1	1.3 ± 0.1	0.6 ± 0.2
Total T ₄ (5.0–12.0 µg/dl)	7.4 ± 0.5	6.5 ± 0.4	2.8 ± 0.6
Free T ₄ (0.8–2.0 ng/ml)	1.0 ± 0.1	0.9 ± 0.2	0.4 ± 0.1

*Patients with chronic autoimmune thyroiditis and subclinical hypothyroidism had anti-thyroidal peroxidase antibodies levels of 2160 ± 1722 U/ml, whereas those with overt primary hypothyroidism had levels of 2685 ± 1350 U/ml. ^aPrior to this study radiotherapy or thyroid surgery had been performed 5–16 months ago in two patients with subclinical hypothyroidism and five patients with overt primary hypothyroidism. ^b30 min after intravenous administration of 200 µg TRH.

the hormone’s metabolic clearance rate, the *in vivo* bioactivity (Grossmann *et al.* 1997a). TSH isoforms bearing terminal sialic acid (sialo-TSH) have a decreased metabolic clearance rate leading to an increased plasma circulation time and therefore to an enhanced biological activity (Szkudlinski *et al.* 1995a). In glycoprotein hormones, sialic acid is preferentially bound to precursors bearing terminal galactose residues (Baenziger & Green 1988). In addition to an increased quantity of TSH an enhanced sialylation of TSH has been observed in hypothyroid animals, which is believed to increase further the *in vivo* bioactivity of TSH (Magner 1990). The enzymes responsible for the coupling of terminal galactose and sialylation of TSH, β -1,4-galactosyltransferase and sialyltransferases, are more strongly expressed in thyrotropes of hypothyroid rats than in euthyroid controls (Helton & Magner 1994a,b).

A major difficulty in analyzing the carbohydrate structures of human serum glycoprotein hormones is the limited amount of available material per individual. Therefore, direct analysis of the carbohydrate content of TSH, which constitutes 20–35% of its weight, is not possible. To overcome this problem, lectins are widely used to characterize the oligosaccharides of serum glycoproteins by affinity chromatography (Gesundheit *et al.* 1987, Kobata & Endo 1992). *Ricinus communis* (RCA 120) is a lectin that binds glycoproteins with exposed galactose (Gal) residues, but does not bind to N-acetylgalactosamine or sulfate (Lin & Li 1980). Neuraminidase cleaves sialic acid from glycoproteins, exposing more Gal residues and causing an increase in ricin binding (Miura *et al.* 1989). To date, there have only been two studies analyzing the degree of sialo-TSH in patients with overt primary hypothyroidism (Miura *et al.* 1989, Papandreou *et al.* 1993). Furthermore, there is no study on TSH sialylation in patients with

subclinical hypothyroidism. In a recent study, Schaaf *et al.* (1995) used the lectin Lentil to further characterize the content of core fucose-bearing TSH isoforms in euthyroid and subclinical hypothyroid patients. They demonstrated a substantial shift in core fucose-bearing TSH isoforms in subclinical hypothyroidism. Magner *et al.* (1992a) have shown that in patients with TSH-secreting pituitary adenomas the degree of core fucosylated TSH tends to be high when sialylation is low and vice versa.

The aim of this work was to further study serum sialo-TSH, its precursors bearing terminal galactose, and the role of pharmacological doses of thyrotropin-releasing hormone (TRH) on the secretion of these TSH isoforms in patients with different degrees of primary hypothyroidism.

Materials and Methods

Subjects

Serum samples were obtained from euthyroid volunteers (*n*=12, 9 females, 3 males, aged 37 ± 4 years), patients with subclinical (*n*=13, 7 females, 2 males, aged 34 ± 4 years) and overt primary hypothyroidism (*n*=12, 10 females, 3 males, aged 46 ± 1 years). The hypothyroid metabolic state was caused by chronic autoimmune thyroiditis in five patients with subclinical and in six patients with overt primary hypothyroidism. Two patients with subclinical hypothyroidism and five patients with overt primary hypothyroidism had undergone radiotherapy or thyroid surgery for Grave’s disease. The patients’ profiles are summarised in Table 1. Blood was drawn before and 30 min after intravenous (i.v.) administration of 200 µg TRH (Relefact, Hoechst, Frankfurt, Germany). In addition, human standard pituitary TSH

(IRP 80/558, National Institute for Biological Standards and Controls, Potters Bar, Herts, UK) was studied. Written informed consent was obtained from each patient and the investigation was approved by the local ethical committee.

Immunoaffinity purification of human serum TSH

TSH from serum samples (10 ml) was purified with anti-hTSH 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, pooled, and concentrated on the Centriprep ultrafiltration system (Amicon, Witten, Germany).

Ricinus communis (RCA 120) affinity chromatography

For cleavage of sialic acid residues, a TSH aliquot was incubated with 10 mIE neuraminidase type X (Sigma, Deisenhofen, Germany), dissolved in citrate buffer (100 mmol/l, pH 6.6), for 4 h at 37 °C. Before *Ricinus communis* affinity chromatography (RCA 120) (Sigma) native TSH and neuraminidase-treated TSH were diluted with 200 µl phosphate-buffered saline (PBS) and the column was washed with 30 ml PBS buffer, pH 7.4, containing sodium azide (1 g/l) and bovine serum albumin (1 g/l). Unbound material was washed off the column with 10 ml PBS buffer and bound material was subsequently eluted with PBS buffer, containing α -D-galactose (200 mmol/l). TSH was measured in 1 ml fractions with an automated TSH immunoassay. The content of TSH isoforms bearing exposed, terminal galactose residues (Gal-TSH) was calculated as the binding ratio of native TSH. The content of asialo-Gal-TSH was calculated as the binding ratio of neuraminidase-treated TSH. The binding difference of native and digested TSH represents the degree of sialo-TSH (Gesundheit *et al.* 1986).

Immunometrical assays

Serum levels of total tri-iodothyronine (T₃), total thyroxine (T₄), free thyroxine (FT₄), and anti-thyroidal peroxidase antibodies (anti-TPO) 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 chorionic gonadotropin was less than 1%. To rule out the possibility that this assay does not recognise all TSH isoforms, TSH isoforms isolated by lectin affinity chroma-

tography 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

Unless otherwise stated data are shown as means $\pm$ S.E.M. Statistical analysis was performed with the Student's *t*-test and significance was assigned to two-tailed *P* values less than 0.05 vs TSH from euthyroid individuals, before or 30 min after i.v. administration of 200 µg TRH.

Results

Serum TSH, purified from blood samples obtained before and 30 min after i.v. administration of 200 µg TRH (TRH-TSH), was analyzed from patients with subclinical and overt primary hypothyroidism and euthyroid individuals. The recovery for basal TSH by the immunoaffinity purification method was $74.4 \pm 6.5\%$ and for TRH-released TSH it was $78.6 \pm 7.4\%$. The reproducibility of TSH binding to *Ricinus communis* was confirmed by nine independent runs using 5–35 µIE pituitary TSH. The recovery of native TSH was $86.4 \pm 1.2\%$ and that of neuraminidase-treated TSH was $80.3 \pm 3.6\%$.

Ricin binding of native TSH

TSH oligosaccharide residues were further characterized with *Ricinus communis* affinity chromatography. As measured by ricin binding of native TSH, human pituitary TSH contained $16.5 \pm 0.8\%$ Gal-TSH. Serum TSH from euthyroid individuals contained $14.6 \pm 1.9\%$ Gal-TSH, whereas the proportion of serum Gal-TSH in patients with subclinical and overt primary hypothyroidism was $23.9 \pm 3.5\%$ ($P < 0.05$ vs euthyroid individuals) and $21.1 \pm 1.7\%$ respectively (Fig. 1). After TRH stimulation Gal-TSH represented $14.2 \pm 1.9\%$ of total serum TSH in euthyroid individuals, $23.0 \pm 2.7\%$ in patients with subclinical, and $19.0 \pm 1.6\%$ in patients with overt primary hypothyroidism. These values did not differ significantly from the proportions of basal TSH (Fig. 2).

Ricin binding of neuraminidase-treated TSH

Ricin-binding of TSH from all patients studied and from human pituitary extracts was increased after digestion with the sialic acid residue-cleaving enzyme neuraminidase ($P < 0.05$ vs native TSH). After neuraminidase digestion,

Figure 1 *Ricinus communis* (RCA 120) affinity chromatography of native pituitary standard TSH (IRP-TSH) and basal serum TSH in euthyroid (EU, $n=12$), subclinical (SPH, $n=9$) and overt primary hypothyroid patients (OPH, $n=13$). (a) Analysis of the ratio of terminally galactosylated (Gal)-TSH, (b) of neuraminidase-treated, terminally galactosylated (asialo-Gal)-TSH, and (c) determination of sialylated (sialo)-TSH. TSH was measured in duplicate with the ACS TSH assay. Data are expressed as means $\pm$ S.E.M. binding to *Ricinus communis*. For pituitary TSH the means $\pm$ S.E.M. binding of three independent runs are shown. * $P<0.05$, *** $P<0.001$ vs TSH from euthyroid individuals (Student's t -test).

Figure 2 *Ricinus communis* (RCA 120) affinity chromatography of TRH-released serum TSH in euthyroid individuals (EU, $n=12$) and patients with subclinical (SPH, $n=9$) and overt primary hypothyroidism (OPH, $n=13$). (a) Analysis of the ratio of terminally galactosylated (Gal)-TSH, (b) of neuraminidase-treated, terminally galactosylated (asialo-Gal)-TSH, and (c) determination of sialylated (sialo)-TSH. Serum was obtained 30 min after i.v. administration of 200 μ g TRH. TSH was measured in duplicate with the ACS TSH assay. Data are expressed as means $\pm$ S.E.M. binding to *Ricinus communis*. ** $P<0.01$, *** $P<0.001$ vs TSH from euthyroid individuals (Student's t -test).

human pituitary TSH contained $23.8 \pm 0.6\%$ asialo-Gal-TSH, whereas this proportion was $35.7 \pm 4.2\%$ for TSH in euthyroid individuals. After neuraminidase treatment the mean binding of TSH from subclinical and overt hypothyroid patients was $48.0 \pm 3.3\%$ and $61.5 \pm 3.8\%$ respectively. Compared with euthyroid individuals the proportion of asialo-TSH in patients with overt primary hypothyroidism was significantly increased ($P < 0.001$) (Fig. 1). After administration of TRH the proportion of asialo-TSH was $30.6 \pm 2.7\%$ in euthyroid individuals, $48.3 \pm 3.7\%$ in patients with subclinical hypothyroidism ($P < 0.01$ vs euthyroid individuals), and $58.6 \pm 3.1\%$ in patients with overt primary hypothyroidism ($P < 0.001$ vs euthyroid individuals) (Fig. 2).

Determination of sialo-TSH

The proportion of sialo-TSH was calculated from the binding difference of native and neuraminidase-treated TSH. For pituitary TSH the calculated proportion of sialo-TSH was $6.5 \pm 0.2\%$. TSH in euthyroid patients contained $20.3 \pm 2.8\%$ sialo-TSH before and $16.6 \pm 3.4\%$ 30 min after i.v. administration of TRH. The mean proportion of sialo-TSH in patients with subclinical hypothyroidism was $24.1 \pm 3.0\%$, whereas this proportion was $40.7 \pm 3.0\%$ in overt primary hypothyroid patients ($P < 0.001$ vs euthyroid individuals) (Fig. 1). After TRH administration the proportion of sialo-TSH in patients with subclinical and overt primary hypothyroidism was $25.1 \pm 4.0\%$ and $38.9 \pm 2.9\%$ ($P < 0.001$ vs euthyroid individuals) respectively (Fig. 2).

Discussion

Sialylation of human serum TSH, its precursors bearing terminal galactose residues, and the role of pharmacological doses of TRH on their secretion have not yet been analyzed sufficiently. We studied serum TSH in patients with subclinical and overt primary hypothyroidism because sialylation of TSH seems to have an important impact on the hormone's *in vivo* bioactivity due to protection from carbohydrate-specific hepatic receptor-mediated clearance mechanisms (Szkudlinski *et al.* 1995b). In addition, sialylation is a major factor affecting the charge heterogeneity of TSH (Szkudlinski *et al.* 1993), which is believed to cause steric changes in the three-dimensional structure of TSH and thus influence the hormone's ability to stimulate the TSH receptor. In accordance with this, it has been demonstrated that certain human TSH glycosylation variants activate inositol phosphate and cAMP signal transduction pathways to different degrees (Schaaf *et al.* 1997).

As direct analysis of the carbohydrate content of individual human serum TSH, as described for human pituitary glycoprotein hormones (Baenziger & Green 1988), is

not possible we chose *Ricinus communis*, a lectin that binds oligosaccharides with exposed, terminal galactose. *Ricinus communis* affinity chromatography in combination with neuraminidase, an enzyme that cleaves sialic acid residues from oligosaccharide chains, is helpful in studying the degree of TSH sialylation (Gesundheit *et al.* 1986). In order to compare our results with previously published work, we have chosen identical conditions to cleave sialic acid residues from TSH oligosaccharides by means of neuraminidase treatment (Miura *et al.* 1989, Magner *et al.* 1992a, Papandreou *et al.* 1993). In addition, we feel that the chosen digestion time of 4 h might overcome incomplete cleavage of sialic acid due to possible different kinetics concerning the hydrolyzation of $\alpha 2,3$ - and $\alpha 2,6$ -linked sialic acid residues of TSH. TSH was measured with a highly sensitive immunoassay that does not discriminate between different TSH isoforms (data not shown). TSH values measured with two TSH assays, based on different anti-hTSH antibodies, were similar. Using these methods we can report the following results: compared with euthyroid individuals, patients with subclinical hypothyroidism have an enhanced proportion of serum Gal-TSH, but not of serum sialo-TSH. However after TRH stimulation, the proportion of serum sialo-TSH was also significantly increased in subclinical hypothyroid individuals. In contrast, patients with overt primary hypothyroidism had a markedly increased proportion of both serum Gal-TSH and sialo-TSH before and after TRH administration. Human pituitary-derived TSH, representing a mixture of more or less mature isoforms, contained a lower degree of both Gal- and sialo-TSH than serum TSH in all individuals studied.

During the post-translational processing of TSH its oligosaccharide chains are added and modified. Although a quite late event in this pathway, the addition of Gal versus N-acetylgalactosamine is a key branching point: oligosaccharide chains containing exposed galactose residues may be further modified by attaching terminal sialic acid, whereas N-acetylgalactosamine residues may become sulfated (Baenziger & Green 1988). The cellular mechanisms causing an increased proportion of secreted Gal- and sialo-TSH in hypothyroid rats were examined by Helton and Magner (1994a,b). Using *in situ* hybridisation they demonstrated that the levels of messenger RNA of the Golgi enzymes involved – namely β -1,4-galactosyltransferase and β -galactoside α -2,6-sialyltransferase – are elevated. To date, the expression of these transferases has not been studied in subclinical hypothyroidism or mild hypothyroidism. Interestingly, we found an enhanced proportion of serum Gal-TSH, but not of serum sialo-TSH, in subclinical hypothyroid patients. Therefore, we speculate that the responsible enzyme β -1,4-galactosyltransferase is already activated in subclinical hypothyroidism. Assuming that the increased proportion of TSH isoforms with terminal galactose implies enhanced TSH sialylation and that the activation of

galactosyltransferase is followed by the activation of sialyltransferases, the presence of an increased proportion of serum Gal-TSH could turn out to be a marker for the progression of hypothyroidism. Our findings are in accordance with others who have demonstrated an increased proportion of serum sialo-TSH in hypothyroidism compared with euthyroid controls (Miura *et al.* 1989, Papandreou *et al.* 1993). Miura *et al.* (1989) found no correlation between the sialo-TSH proportion and serum TSH level, but patients with hypothyroidism ongoing for more than 1 year had a higher sialo-TSH proportion than those who had been hypothyroid for less than 3 months. Recently, Magner and co-workers (1997) reported a case of decreased TSH sialylation in a patient with euthyroid sick syndrome, which may account for the altered TSH bioactivity that has been described in such patients.

In euthyroid individuals, the i.v. administration of TRH releases TSH containing more core fucose residues than basal TSH (Magner *et al.* 1992b). We found no statistical difference in TRH-released Gal-TSH, asialo Gal-TSH, and sialo-TSH in euthyroid individuals or in patients with subclinical or overt hypothyroidism compared with basal TSH. In a recent publication, Schaaf *et al.* (1995) showed an enhanced core fucosylation of TRH-released TSH in patients with subclinical hypothyroidism compared with euthyroid individuals. In the present study, we found a substantial increase in the proportion of sialo-TSH in patients with overt hypothyroidism, but not in those with subclinical hypothyroidism. This might explain the findings of Schaaf *et al.* (1995) who failed to show a difference in the net charge between serum TSH in euthyroid and subclinical hypothyroid individuals, suggesting a similar proportion of negatively charged TSH isoforms. Grossmann and colleagues (1997b) demonstrated that recombinant TSH, expressed in insect cells, lacks complex-type oligosaccharides terminating with sialic acid but contains predominantly high mannose-type oligosaccharides. This resulted in an increased *in vitro* bioactivity but, due to rapid metabolic clearance, in a much lower *in vivo* activity compared with recombinant TSH, expressed in Chinese hamster ovary cells (Szkudlinski *et al.* 1993). These data support the hypothesis that glycosylation of recombinant hTSH is dependent on the cell system used and is most likely due to different expression of Golgi enzymes. *In vivo* biological activity of TSH is crucial, especially for the use of recombinant human TSH in stimulating radioiodine uptake for radioactive iodine scanning in patients with thyroid cancer (Ladenson *et al.* 1997).

In summary, the present study confirmed an enhanced proportion of terminally sialylated serum TSH isoforms in patients with overt primary hypothyroidism. In contrast to overt hypothyroidism, patients with subclinical hypothyroidism have an unchanged proportion of sialylated TSH isoforms, but the proportion of TSH isoforms with terminal galactose, representing precursors of sialo-TSH,

was enhanced. Pharmacological doses of TRH cause an increased quantity of TSH to be released, but do not significantly alter the proportion of sialylated or terminally galactosylated TSH isoforms. The physiological roles of TSH isoforms are not precisely understood yet, but the modulation of TSH heterogeneity by regulation of glycosyltransferases may be a mechanism to stimulate further thyroid function.

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