Human Thyrotropin-Releasing Hormone-Associated Peptide 3 (hTAP-3) in Serum

MASANOBU YAMADA, SHUNTARO KONAKA, TETUROU SATOH, TSUYOSHI MONDEN AND MASATOMO MORI

First Department of Internal Medicine, Gunma University School of Medicine, Maebashi, Gunma 371, Japan

Abstract. Human thyrotropin-releasing hormone (TRH)-associated peptide 3 (hTAP-3), one of the cryptic peptides resulting from the proteolytic processing of preproTRH to produce TRH, was measured in human plasma from normal, hyperthyroid, and hypothyroid subjects. The dilution curve of hTAP-3 immunoreactivity in the serum paralleled the standard curve of the radioimmunoassay. HPLC analysis revealed a single strong immunoreactive peak, which corresponded to the authentic peptide, hTAP-3. The half-life of hTAP-3 in serum was approximately 3.5 min, and the addition of aprotinin and EDTA completely prevented its degradation. In hyperthyroid patients, plasma concentrations of hTAP-3 were significantly higher than those in the control group and hypothyroid patients, but no correlation was found between its level and that of thyroid hormone. These findings indicate the existence of intact hTAP-3 in the human serum and increases in plasma hTAP-3 levels in hyperthyroid patients, suggesting that blood hTAP-3 may be derived from the peripheral organs rather than the hypothalamus.

Key words: hTAP-3, TRH, Cryptic peptide

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THYROTROPIN-releasing hormone (pGlu-His-ProNH₂, TRH) is a major stimulator of synthesis and secretion of thyrotropin (TSH) in the anterior pituitary [1, 2]. Similarly to other peptide hormones, TRH is derived from a large precursor peptide, preproTRH [3]. Cloning of the human preproTRH gene and hypothalamic cDNA by us revealed that human preproTRH is 242 amino acids in length and has a characteristic structure containing 6 repetitive copies of the TRH progenitor sequence (Lys-Arg-Gln-His-Pro-Gly-Lys/Arg-Arg) [4]. We designated the intervening peptides between these TRH progenitor sequences as human TRH-associated peptides 1–5 (hTAP1–5) [5]. hTAP-3 is an intervening peptide containing 9 amino acids

(SPTLAYAVP) located between the 3rd and 4th TRH progenitor sequences and corresponds to 160–169 amino acids of the rat preproTRH (Ps4). It has recently been reported that rat Ps4 has significant biological activity including stimulation of TRH-induced TSH and PRL release and increases in these mRNA levels in the anterior pituitary [6–9].

We recently established radioimmunoassay (RIA) systems specific for five intervening peptides of human preproTRH including hTAP-3 [5]. Using these systems, we demonstrated the existence of hTAPs in the human placenta and a difference in processing pattern of preproTRH between the hypothalamus and placenta. As expression of the TRH gene in the hypothalamus is negatively regulated by thyroid hormones, serum concentrations of hTAPs may be altered in patients with different thyroid status [10–12]. In the present study, we first characterized hTAP-3 immunoreactivity in human serum and compared results among hyperthyroid, hypothyroid and normal subjects.

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Correspondence to: Masanobu YAMADA M.D., Ph.D. First Department of Internal Medicine Gunma University School of Medicine, 3–39–15 Showa-cho, Maebashi 371–8511, Japan

Materials and Methods

Radioimmunoassay

The RIA system used in this study was developed previously in our laboratory [5]. Cross-reactivity of rabbit anti-human TAP-3 antibody against other intervening peptides, TRH and TRH-Gly was less than 0.1%, and intra- and inter-assay CV were less than 10%. To characterize hTAP-3 immunoreactivity in human serum, we compared its dilution curve with the standard curve of authentic hTAP-3 in our RIA system. We next determined the degradation rate of hTAP-3 in the human serum by incubation of serum at 37°C for various periods and measurement of hTAP-3 immunoreactivity. In addition, for the subsequent experiment, we also examined whether aprotinin and EDTA used for measurement of

glucagon was effective to prevent the degradation of hTAP-3 in serum.

High performance liquid chromatography

To concentrate hTAP-3 immunoreactivity in human serum, aliquots of 5 ml of human serum were applied to Sep-Pak C18 cartridges (Waters Millipore, MA) and eluted with 60% acetonitrile. After removing the acetonitrile by evaporation, the precipitate was suspended in 0.1% TFA and subjected to reversed phase high performance liquid analysis (HPLC) (Hitachi) with a Sep-Pak C18 column and a gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid (elution speed, 0.37 ml/min, fraction, 1 min/tube). Each sample was evaporated, the remaining precipitate was resuspended in 1% BSA-PBS (RIA-buffer), and hTAP-3 immunoreactivity was measured as described above.

Fig. 1. a) Dilution curve of hTAP-3 immunoreactivity in human serum. b) Reversed phase HPLC analysis of concentrated human serum. After concentration of 5 ml aliquots of human serum with Sep-Pak 18 columns, the eluted samples were subjected to HPLC. Squares represent the profile of immunoreactivity for authentic hTAP-3 and triangles indicate human serum.

Subjects

To determine the effects of thyroid status on concentrations of hTAP-3 immunoreactivity in human plasma, we measured plasma hTAP-3 from patients with hyperthyroidism (Graves' disease), primary hypothyroidism, and normal subjects. Subjects included 11 untreated hyperthyroid patients, 7 hypothyroid patients, and 14 normal subjects. These groups included 5, 3, and 9 men, and 6, 4, and 5 women, respectively. The mean ages of the groups were 37.5 ± 4.0 , 41.0 ± 13.0 and 33.2 ± 5.5 , respectively.

Statistical analysis

All data were analyzed by analysis of variance (ANOVA) and Duncan's multiple range test.

Results

Characterization of hTAP-3 immunoreactivity in human serum

As shown in Fig 1a, the curve of serial dilution of hTAP-3 immunoreactivity in the human serum paralleled authentic hTAP-3 (Fig. 1a). HPLC analysis of concentrated human serum showed a strong immunopositive signal at the same position as

Fig. 2. The half-life of hTAP-3 immunoreactivity in human serum and prevention of its degradation by addition of aprotinin and EDTA.

authentic hTAP-3 (Fig. 1b). These observations suggested the existence of intact hTAP-3 in human serum. This hTAP-3 detected in the human serum was degraded relatively rapidly, and its half-life was approximately 3.5 min (Fig. 2). The degradation was completely blocked by 500 U/ml aprotinin and 0.5 mg/ml EDTA even after incubation at 37°C for 15 min.

Effects of thyroid status on hTAP-3 immunoreactivity in human plasma

Using the above blocking reagents, hTAP-3 immunoreactivity was measured in subjects with different thyroid status. Mean serum free T4 levels in hyper- and hypothyroid patients were 5.48 ± 1.0 , and 0.47 ± 0.29 ng/dl (means $\pm$ S.E.) (normal, 0.81–2.13), and TSH levels were <0.1 and 161.4 ± 89.0 μ U/ml, respectively (normal, 0.5–5.5). There were no sex differences of plasma hTAP-3 concentration in each group. Mean concentration of hTAP-3 was significantly higher in hyperthyroid patients (2.23 ± 0.12 ng/ml, means $\pm$ S.E.) than those in normal subjects (1.54 ± 0.06) and hypothyroid patients (1.57 ± 0.14) ($p < 0.001$) (Fig. 3). In contrast, there were no significant differences between hypothyroid patients and normal subjects. Furthermore, no correlations were found between plasma hTAP-3 and

Fig. 3. Concentrations of plasma hTAP-3 in subjects with different thyroid status.

Table 1. Plasma hTAP-3 immunoreactivity, and serum free T4 and T3 levels in patients with hyperthyroidism.

No.	Patients	Sex	Age	Free T3 (pg/dl) (2.6–5.4)	Free T4 (ng/dl) (0.81–2.13)	hTAP-3 (ng/ml)
1.	S.M.	M	48	13.5	12.8	2.10
2.	S.O	F	16	88.1	5.8	2.91
3.	K.H	F	42	8.3	2.2	2.60
4.	N.T	F	40	36.9	5.8	1.68
5.	H.M	M	35	17.4	4.3	2.27
6.	M.K	M	41	8.8	2.6	2.52
7.	H.N	F	34	41.3	10.0	1.72
8.	S.H	M	31	17.1	6.8	1.97
9.	S.O	F	68	10.5	3.3	2.00
10.	T.M	M	28	8.3	3.1	2.55
11.	K.K	F	29	9.8	3.6	2.25

thyroid hormone levels in hyperthyroid patients ($r=0.19$ with free T3, $r=-0.46$ with free T4) (Table 1).

Discussion

In the present study, we demonstrated for the first time the presence of hTAP-3 immunoreactivity in human serum, and HPLC analysis suggested that it may be in intact form. Most of hTAP-3 in human serum degraded within 15 min, but the degradation was completely blocked in the presence of EDTA and aprotinin. hTAP-3 corresponds to amino acids 160–169 of the rat preproTRH (Ps4), which has been recently reported to possess significant biological activity mediating TRH-induced stimulation of TSH β gene expression, TSH and prolactin release from the anterior pituitary [6–9]. Specific receptors for Ps4 in the rat anterior pituitary have also been reported recently [13–15]. Although it remains to be elucidated whether hTAP-3 possesses the same activity as rat Ps4, the presence of intact hTAP-3 in human serum suggested that serum hTAP-3 may be involved in regulation of pituitary function.

TRH and the intervening peptides of preproTRH are distributed in many organs such as the pancreas, gastrointestinal tract, and placenta [2, 5, 16, 17]. In these organs, preproTRH mRNA is also expressed [5]. These observations suggested that preproTRH is produced in the peripheral organs, and that after processing TRH and intervening peptides are secreted

into the circulating blood. Synthesis of hypothalamic TRH has been reported to be negatively regulated by thyroid hormone through the negative thyroid hormone response element (nTRE) in the promoter region of the preproTRH gene [11]. If circulating TRH and intervening peptides are mainly derived from the hypothalamus, these concentrations may reflect thyroid hormone status. However, our results did not support this hypothesis and showed that plasma hTAP-3 concentration in the hyperthyroid patients was paradoxically increased rather than decreased. Several previous studies showed that the origin of circulating TRH may be the peripheral organs rather than the tuberoinfundibular system [18, 19]. Taken together with the present results, these observations suggested that serum hTAP-3 may also be derived mainly from peripheral organs rather than the hypothalamus.

It has been reported that the amounts of TRH and intervening peptides of preproTRH is regulated by preproTRH biosynthesis and metabolism by specific convertases and endopeptidases, some of which have been shown to be stimulated by thyroid hormone [18–20]. Therefore, the increased levels of plasma hTAP-3 in hyperthyroid patients may be the result of increased rate of conversion of preproTRH towards hTAP-3 or an increase in the preproTRH gene expression in peripheral organs. However, the plasma hTAP-3 levels in hypothyroid patients were similar to those in normal subjects, suggesting a decrease in the degradation rate of hTAP-3 or that factors other than thyroid hormone level may be involved in regu-

lation of blood hTAP-3 level. Although further studies are required to determine the precise mechanism by which serum hTAP-3 concentration

was increased in patients with hyperthyroidism, plasma hTAP-3 level may be a new parameter for evaluating hyperthyroid status in humans.

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