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Evidence that the TRH-like peptide pyroglutamyl-glutamyl-prolineamide in human serum may not be secreted by the pituitary gland

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Abstract

Recent studies have revealed that TRH-like immunoreactivity (TRH-LI) in human serum is predominantly *p*Glu-Glu-ProNH₂ (<EEP-NH₂), a peptide previously found in, among others tissues, the pituitary gland of various mammalian species. In the rat pituitary, <EEP-NH₂ is present in gonadotrophs and its pituitary content is regulated by gonadal steroids and gonadotrophin-releasing hormone (GnRH). Hence, we reasoned that <EEP-NH₂ in human serum may also arise, at least in part, from the pituitary, and that its secretion may correlate with that of gonadotrophins. Therefore, blood was simultaneously sampled from both inferior petrosal sinuses, which are major sites of the venous drainage of the pituitary gland, and a peripheral vein from seven patients with suspected adrenocorticotrophin-secreting pituitary tumours. In addition, in six postmenopausal and six cyclic women, peripheral vein blood was collected at 10-min intervals for 6 h, then a standard 100 µg GnRH test was performed. In the sera, TRH-LI was estimated by RIA with antiserum 4319, which binds most tripeptides that share the N- and C-terminal amino acids with TRH (*p*Glu-His-ProNH₂). In addition, LH and FSH were measured in these sera by RIA. In the blood samples taken at 10-min intervals, an

episodic variation in serum TRH-LI was noted and pulses of TRH-LI were detected at irregular intervals (from one to six pulses per 6 h) in five postmenopausal and six cyclic women. In general, these pulses did not coincide with those of LH and FSH, suggesting that TRH-LI is not co-secreted with gonadotrophins. Moreover, unlike LH and FSH, serum TRH-LI did not increase during the menopause or after exogenous administration of GnRH. Whereas gonadotrophin concentrations were significantly greater in the inferior petrosal sinus than in peripheral serum, there were no differences in TRH-LI concentrations between these serum samples. In conclusion, serum TRH-LI in humans seems not to be regulated by gonadal steroids or GnRH. Moreover, serum derived directly from the pituitary contained no more TRH-LI than did peripheral serum, which suggests that the human pituitary gland does not secrete significant amounts of <EEP-NH₂, and therefore does not contribute significantly to serum TRH-LI concentrations. Further research is required to identify the site of origin of <EEP-NH₂ in human serum.

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Introduction

Thyrotrophin-releasing hormone (TRH) was originally isolated from hypothalamic extracts. Subsequently, TRH-like immunoreactivity (TRH-LI) was detected in peripheral tissues, blood and urine (for reviews, see Hökfelt *et al.* 1989, Ashworth 1994, Iversen 1995). Although TRH-LI in the brain and certain peripheral tissues has been identified as authentic TRH (Pekary *et al.* 1980, 1993, Ashworth 1994, Iversen 1995, Klootwijk *et al.* 1997a), TRH-LI in other tissues is attributable to peptides that are

structurally related to TRH (Cockle *et al.* 1989, Khan *et al.* 1992, Ashworth 1994, Gkonos *et al.* 1994, Cockle 1995, Rondeel *et al.* 1995a,b, Linden *et al.* 1996). These TRH-like peptides, which differ from TRH (*p*Glu-His-ProNH₂) in the middle amino acid, cross-react to varying extents with antisera raised against TRH (Pekary *et al.* 1993, Rondeel *et al.* 1995a, Klootwijk *et al.* 1995, 1996). The knowledge of the function of these TRH-like peptides remains limited, but physiological actions have been reported for *p*Glu-Glu-ProNH₂ (<EEP-NH₂), a peptide detected first in the prostate gland (Cockle *et al.*

Table 1 Characteristics of TRH-LI, LH and FSH in serum of postmenopausal ($n=6$) and premenopausal ($n=6$) women. Blood was taken from these volunteers at 10-min intervals for 6 h. Premenopausal women were studied at day 3 of the menstrual cycle. Results are means \pm S.E.M.

Variable	Postmenopausal	Premenopausal
TRH-LI		
Mean concentration (pg/ml)	16.4 \pm 1.7	21.6 \pm 3.0
Mean amplitude (pg/ml)	13.3 \pm 1.3	17.1 \pm 2.4
Pulses per 6 h	2.7 \pm 1.0	3.3 \pm 0.7
LH		
Mean concentration (mU/ml)	19.2 \pm 3.1	3.2 \pm 0.8*
Mean amplitude (mU/ml)	4.1 \pm 0.4	1.1 \pm 0.3*
Pulses per 6 h	4.3 \pm 0.3	4.5 \pm 0.3
FSH		
Mean concentration (mU/ml)	68.1 \pm 11.4	7.3 \pm 1.7*
Mean amplitude (mU/ml)	6.4 \pm 1.5	1.0 \pm 0.3*
Pulses per 6 h	4.7 \pm 0.7	3.2 \pm 0.5

* $P < 0.05$ compared with postmenopausal women.

1989) and later in semen and the anterior pituitary gland of various mammalian species (for reviews, see Ashworth 1994, Cockle 1995), in a human neuroblastoma cell line (Rondeel *et al.* 1994) and in serum and urine of patients with metastatic carcinoid tumours (Klootwijk *et al.* 1996, 1997b). Recently, <EEP-NH₂ has been found to enhance the fertilizing ability of human and mouse sperm (Green *et al.* 1994, 1996a), to inhibit the acrosome reaction of mouse sperm (Green *et al.* 1996b), to increase the motility of sperm from certain infertile men (Linden *et al.* 1996), to affect pituitary growth hormone release in domestic fowls and rats (Harvey *et al.* 1993, Ashworth *et al.* 1994, Rondeel *et al.* 1995b) and to inhibit adrenal corticosterone secretion in rats (Neri *et al.* 1993). Because of its effect on sperm, <EEP-NH₂ has also been called *fertilization promoting peptide* (FPP, Cockle 1995).

Serum of healthy humans also contains TRH-LI that is not identical to TRH (Iversen 1986), and recent data show that the predominant TRH-like peptide in human and rat serum is <EEP-NH₂ (Bertram & Cockle 1995, Klootwijk *et al.* 1997a,b). Although prostatic secretion of <EEP-NH₂ may contribute to serum concentrations of TRH-LI in men (Bertram & Cockle 1995, Klootwijk *et al.* 1997b), the similarity of serum TRH-LI in men and women (Klootwijk *et al.* 1996, 1997b) indicates that other sources of <EEP-NH₂ exist. Studies in rats have provided suggestive evidence that <EEP-NH₂ in the pituitary gland is produced by the gonadotrophs (Rondeel *et al.* 1995a,b), and have revealed that the pituitary content of <EEP-NH₂ is influenced by treatments affecting gonadotrophin release (Akinsanya *et al.* 1995, Rondeel *et al.* 1995a,b). We have studied the possibility that, in human serum, <EEP-NH₂ is derived at least in part from hypophysial secretion, and that this secretion is correlated with that of gonadotrophins.

Study participants and methods

All studies were performed according to the rules of the respective medical ethics committees of the University Hospitals of Amsterdam and Rotterdam.

Profiles of TRH-LI and gonadotrophins in peripheral serum of postmenopausal and cyclic women

Twelve healthy women volunteers, six postmenopausal (aged 52–72 years, median 57 years) and six premenopausal women at day 3 of the menstrual cycle (ages 32–38 years, median 36 years), participated in the study. A cannula was placed in a peripheral vein at between 0800 and 0900 h, and blood samples were removed from the cannula every 10 min for 6 h. At the end of the 6 h period, 100 μ g gonadotrophin-releasing hormone (GnRH; Wyeth, Hoofddorp, The Netherlands) was injected through the cannula and additional blood samples were taken 30, 60 and 90 min later. Blood was allowed to clot and serum was kept at room temperature for at least 2 h to ensure the enzymatic degradation of any endogenous TRH-LI that was not identical to <EEP-NH₂ (Cockle *et al.* 1994, Klootwijk *et al.* 1997b) by serum pyroglutamyl aminopeptidase II (Schauder *et al.* 1994). The serum samples were stored at -20°C until required for analysis for TRH-LI, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by RIA.

Concentrations of TRH-LI and gonadotrophins in peripheral and inferior petrosal sinus serum

Synchronous blood samples were obtained from both inferior petrosal sinuses and from a peripheral vein as described previously (de Herder *et al.* 1994), for the

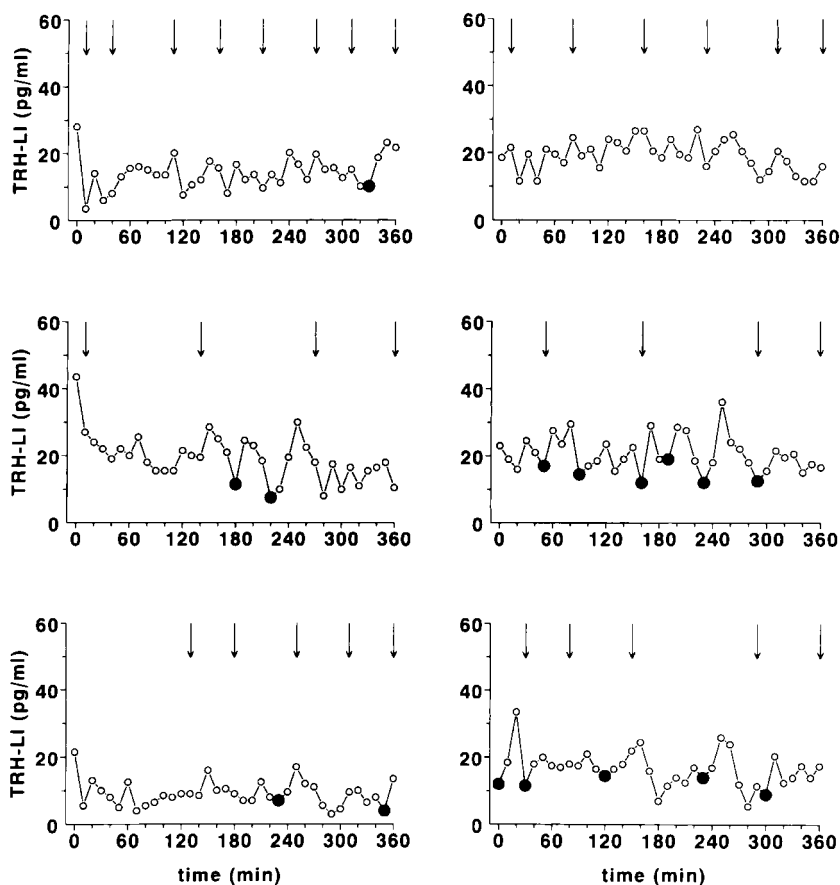


Figure 1 Patterns of serum concentrations of TRH-LI in individual postmenopausal women. Blood samples were taken every 10 min for 6 h. ●, nadir of detected TRH-LI pulses; arrows indicate the occurrence of LH pulses.

diagnosis of pituitary-dependent Cushing's disease in four women (aged 25–73 years, median 40 years) and three men (aged 28–66 years, median 63 years). Blood was removed before (0 min) and several times after stimulation with corticotrophin-releasing hormone (CRH, 1 µg/kg, Bissendorf Peptide Pharmaentwicklung und Vertrieb GmbH, Hannover, Germany) to evaluate the response of adrenocorticotrophin (de Herder *et al.* 1994). On the basis of the resulting serum concentrations of adrenocorticotrophin, four of these patients were found to have Cushing's disease. Retrospectively, it was found that two patients did not suffer from Cushing's syndrome, but had generalized obesity, and one had an adrenal cortisol-secreting adenoma. The blood samples were treated as described above, and the sera were stored at -20°C until required for analysis.

Assays

All samples from each participant were estimated at least in duplicate in the same assay. Concentrations of TRH-LI

were determined in unextracted serum by RIA with antiserum 4319, an antiserum which detects most TRH-like peptides that differ from TRH by the central amino acid – i.e. peptides with the structure pGlu-X-ProNH₂ (Rondeel *et al.* 1995a, Klootwijk *et al.* 1995, 1996). Antiserum 4319 was used at a final dilution of 1:20 000 in the RIA, using ¹²⁵I-TRH as tracer and unlabelled TRH as standard. The limit of detection for this assay is 3–5 pg TRH/ml, and the intra- and interassay coefficients of variation were less than 15%. Previously, we provided evidence that most TRH-LI in human serum kept at room temperature for at least 2 h is accounted for by <EEP-NH₂ (Klootwijk *et al.* 1997b). In this paper, however, we nevertheless present the data as TRH-LI, expressed as TRH equivalents, because minor contributions from other TRH-like peptides cannot be excluded.

In the samples collected at 10-min intervals, LH and FSH were measured by a double antibody IRMA (IRMA-mat BYK-Sangtec Diagnostica GmbH, Dietzenbach, Germany). In inferior petrosal sinus and corresponding peripheral serum samples, gonadotrophins

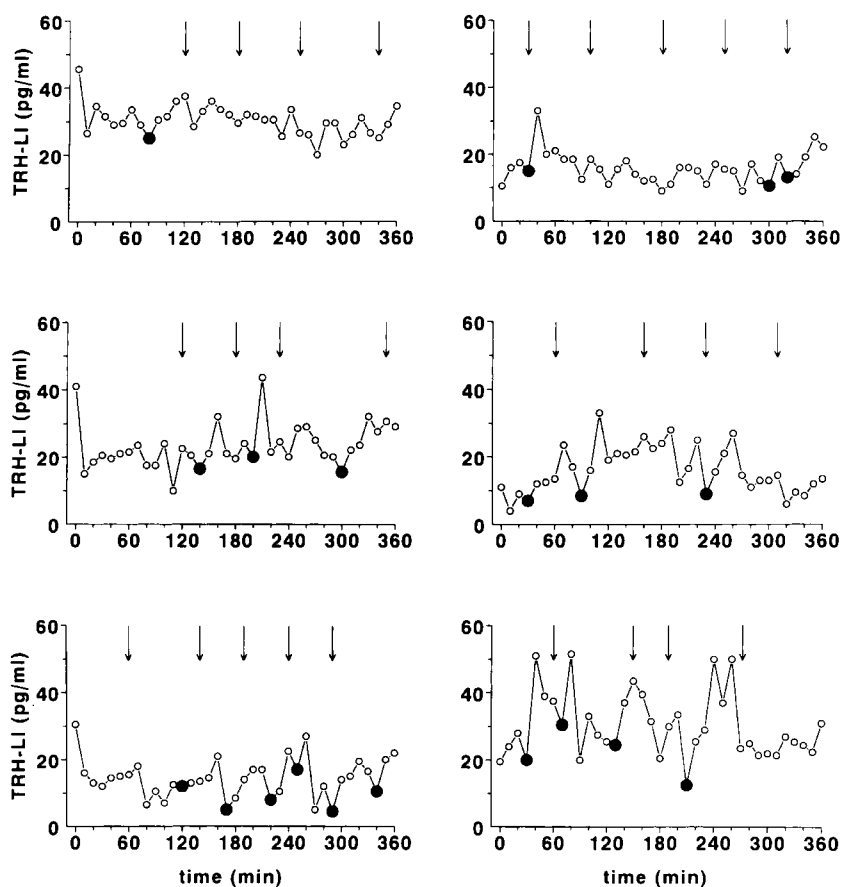


Figure 2 Patterns of serum concentrations of TRH-LI in individual fertile women on day 3 of the menstrual cycle. Blood samples were taken every 10 min for a period of 6 h. ●, nadir of detected TRH-LI pulses; arrows indicate the occurrence of LH pulses.

were estimated using an immunometric method with an enhanced luminescence endpoint (Amerlite; Johnson & Johnson Clinical Diagnostics, Amersham, UK). The lower limits of detection of the gonadotrophin assays were less than 0.2 IU/l, and references for expression of LH and FSH were first IRP 68/40 and second IRP 78/549, respectively; the intra- and interassay coefficients of variation were less than 8%.

Pulse detection of serum TRH-LI, LH and FSH was performed as described previously (Lambalk *et al.* 1985, Scheele *et al.* 1987). The algorithm of the analysis is valid for replicates of repeated measurements with a chance of less than 5% to indicate non-existing pulses as a pulse in a series of 100 samples taken from pooled serum. Nadirs preceding the pulses, rather than the pulses themselves, were used for analyses of the hormone patterns. Pulses of TRH-LI, LH and FSH were considered to be asynchronous when they started more than 10 min apart (Veldhuis *et al.* 1991). The maximum gonadotrophin and TRH-LI increments were taken as the measure of their response to GnRH.

Statistical analyses

Results are given as means \pm s.e.m., and statistical tests included analysis of variance, Wilcoxon matched-pairs signed-ranks test and linear regression analysis. $P \leq 0.05$ was considered significant.

Results

Small but measurable amounts of TRH-LI were detected in the serum samples. Isocratic reverse-phase high performance liquid chromatography revealed that most TRH-LI in serum of normal volunteers co-eluted with <EEP-NH₂ (data not shown). Mean serum concentrations of TRH-LI were found to be similar in cyclic and postmenopausal women, whereas serum concentrations of LH and FSH were greater in postmenopausal than in cyclic women (Table 1). An episodic variation in serum TRH-LI was observed and, in 11 women, pulses of TRH-LI were detected at irregular intervals (Figs 1 and 2). In these women, pulses occurred between one and

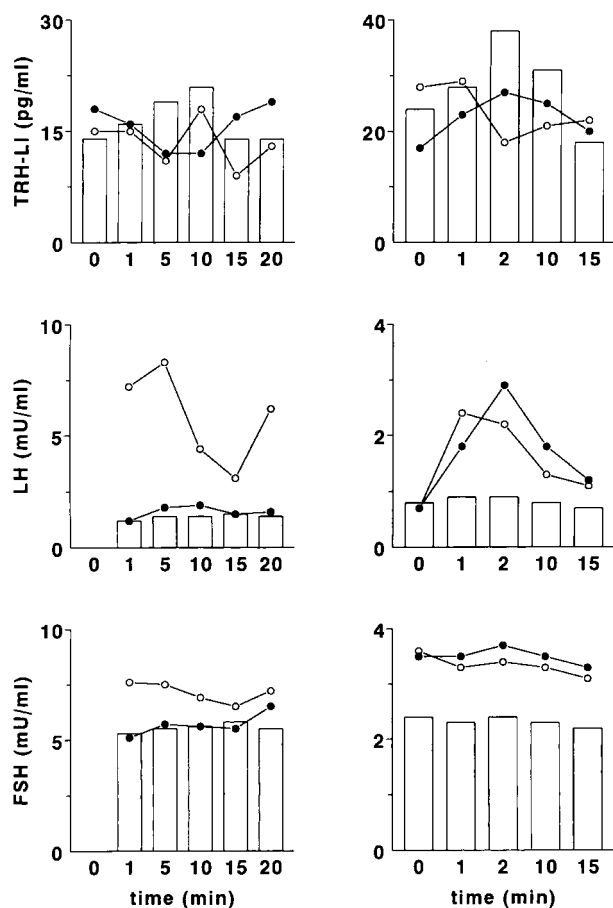


Figure 3 Concentrations of TRH-LI, LH and FSH in serum of two obese patients, obtained by simultaneous blood sampling of a peripheral vein (open bar) and the left (○) and right (●) inferior petrosal sinuses. Left: results from a 63-year-old man. Right: results from a 35-year-old woman using oral contraceptives.

six times during the period of observation, with a mean of about three pulses per 6 h (Table 1). The pulses of TRH-LI in these female volunteers in general did not coincide with pulses of LH (Figs 1 and 2) or FSH (data not shown). Whereas serum concentrations of LH and FSH increased significantly in all women in response to 100 µg GnRH (LH: $376 \pm 28\%$, FSH: $152 \pm 4\%$; $P < 0.01$), no significant effect of GnRH on serum TRH-LI was observed ($122 \pm 16\%$).

As expected, a significant correlation ($r = 0.80$, $P < 0.01$) was found between serum LH and FSH during the 6-h period of observation. In contrast, serum concentrations of TRH-LI and FSH, averaged over the 6-h period, did not correlate significantly ($r = -0.42$; $P = 0.18$). An almost significant inverse correlation was observed between the mean TRH-LI and LH concentrations ($r = -0.55$; $P = 0.06$). Because of this possible negative relation, we evaluated whether peaks of TRH-LI pulses occurred

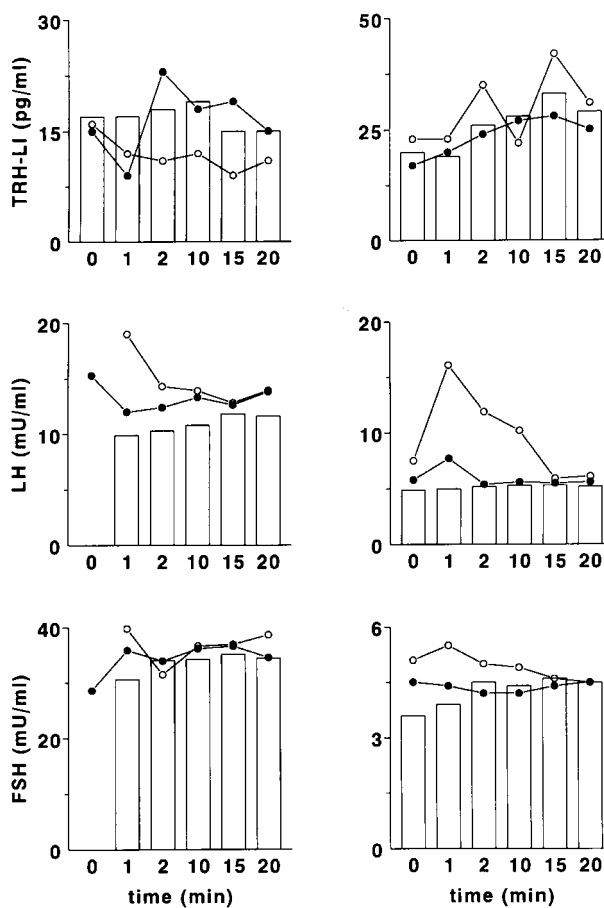


Figure 4 Concentrations of TRH-LI, LH and FSH in serum of two patients with pituitary-dependent Cushing's disease, obtained by simultaneous blood sampling of a peripheral vein (open bar) and the left (○) and right (●) inferior petrosal sinuses. Left: results from a 73-year-old woman. Right: results from a 66-year-old man.

within 10 min of the nadir of an LH pulse; however, this proved to be the case with only six of 36 TRH-LI pulses.

TRH-LI concentrations were found to be similar in serum obtained from the inferior petrosal sinuses or from a peripheral vein of the two obese patients (Fig. 3), the four patients suffering from pituitary-dependent Cushing's disease (Fig. 4 shows data for two of them) and the patient with a cortisol-secreting adenoma (data not shown). During the period of observation, concentrations of TRH-LI in inferior sinus petrosus serum of these seven subjects were $94 \pm 3\%$ of those in peripheral serum. In contrast, concentrations of LH and FSH were significantly greater in inferior sinus petrosus serum than in peripheral serum (LH: $205 \pm 23\%$, FSH: $121 \pm 6\%$; $P < 0.025$). Similar findings were made for growth hormone and prolactin (data not shown), and during the sampling period the concentrations of these hormones in inferior sinus petrosus serum were, respectively, $997 \pm 63\%$ and $470 \pm 99\%$

greater ($P < 0.025$) than the values measured in peripheral serum.

Discussion

This study concerned the presence, in human serum, of TRH-LI, concentrations of which in women were found to exhibit episodic variations. Recent data have revealed that the predominant TRH-LI in serum of normal human subjects is <EEP-NH₂ (Bertram & Cockle 1995, Klootwijk *et al.* 1996, 1997b, present study), and similar findings have been made for rat serum (Klootwijk *et al.* 1997a). Because <EEP-NH₂ in the rat pituitary gland is probably synthesized by gonadotrophin-secreting cells and is regulated in a 'gonadotrophic' manner (Akinsanya 1995, Rondeel *et al.* 1995a,b), we hypothesized that TRH-LI in human serum originates at least in part from the pituitary gland and correlates with the secretion pattern of gonadotrophins. Our objective was to test this hypothesis.

Concentrations of TRH-LI were found to be similar in blood samples simultaneously obtained from both inferior petrosal sinuses, which are major sites of venous drainage of the anterior pituitary gland, and from a peripheral vein. Thus serum derived directly from the pituitary gland does not contain more TRH-LI than peripheral serum. This observation suggests that the pituitary gland does not secrete significant amounts of <EEP-NH₂. Because petrosal sinus sampling was performed in patients with suspected adrenocorticotrophin-secreting pituitary tumours, it cannot be excluded that the presence of such tumours could have resulted in the impaired secretion of TRH-LI from the pituitary gland. However, because LH and FSH concentrations were significantly greater in sinus petrosus serum than in peripheral serum during the period of observation, it seems unlikely that the presence of adenomas prevented the pituitary release of TRH-LI. Moreover, in two obese patients without a tumour, TRH-LI concentrations were similar in sinus petrosus and peripheral serum.

The lack of difference between TRH-LI concentrations in sinus petrosus and peripheral serum may also be explained by a long half-life of <EEP-NH₂ in blood, which would mask potential pituitary secretion of this peptide. In rats, the disappearance of <EEP-NH₂ from serum (Klootwijk *et al.* 1997a) is comparable to that of FSH and LH (de Greef *et al.* 1983), and intravenously injected <EEP-NH₂ could be recovered unchanged and quantitatively in urine (Klootwijk *et al.* 1997a). In humans also, serum <EEP-NH₂ is cleared by renal excretion and its urinary clearance rate is similar to the glomerular filtration rate (Klootwijk *et al.* 1997b). Thus these data suggest that the similar TRH-LI concentrations in sinus petrosus and peripheral serum are not caused by a long half-life of <EEP-NH₂.

We also investigated whether TRH-LI was co-secreted with LH or FSH in healthy female volunteers. Although

serum concentrations of TRH-LI were found to fluctuate, the pulses were not synchronous with those of LH or FSH, but rather, an almost significant inverse correlation was found between the mean TRH-LI and LH concentrations. These findings indicate that, at least in human females, TRH-LI is probably not co-secreted with gonadotrophins.

Because the <EEP-NH₂ content in rat pituitary is affected by both gonadectomy and GnRH (Akinsanya *et al.* 1995, Rondeel *et al.* 1995a,b), we also analysed the effect of menopause and GnRH on serum TRH-LI in women. Although serum concentrations of LH and FSH were greater in postmenopausal than in cyclic women, TRH-LI values were similar in both groups of women. Moreover, whereas administration of GnRH invariably increased serum concentrations of LH and FSH in both cyclic and postmenopausal women, no consistent acute effect of GnRH on serum TRH-LI was observed.

In conclusion, serum TRH-LI was found to exhibit an episodic variation. Although <EEP-NH₂ has been found in the anterior pituitary gland of various mammalian species (Cockle 1995), our observations do not provide evidence that, in humans, hypophysial secretion of <EEP-NH₂ contributes significantly to serum concentrations of TRH-LI. Thus further research is required to identify the site of origin of <EEP-NH₂ in human serum.

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