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Peptides related to thyrotrophin-releasing hormone (TRH) in human prostate and semen

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Abstract

The TRH-related peptide, pGlu-Glu-ProNH$_2$, which was first identified in rabbit prostate has recently been named fertilization-promoting peptide (FPP) because of its ability to enhance the in vitro fertilizing potential of mouse epididymal spermatozoa. This study set out to examine the nature of the TRH-related peptides in human prostate and semen but, first, the optimal conditions for collection of semen samples were investigated. FPP was degraded slowly ($t_{1/2} = 163$ min, S.E. $\pm$ 51.3, $n = 6$) in seminal plasma which has allowed us to measure accurately the concentrations of FPP, after extraction of the peptide in acidified acetone precisely 5 min after ejaculation. In this way, high levels of FPP (mean: 49.5 nmol/l) were detected in normal human semen, from young men, although other TRH-related peptides did not appear to be present. We have also examined the TRH-related peptides present in prostate samples from clinical patients both with and without evidence of benign prostatic hyperplasia (BPH), by ion-exchange chromatography followed by radioimmunoassay. Substantial concentrations of FPP were observed in normal (4.10 pmol/g tissue, S.E. $\pm$ 1.46) and BPH prostate (6.27 pmol/g tissue, S.E. $\pm$ 1.65). In addition, a second, neutral TRH-immunoreactive peptide was always detected in BPH tissue (7.40 pmol/g tissue, S.E. $\pm$ 1.98) with only low levels generally present in normal prostate. The possibility that the presence of high levels of the neutral peptide in prostate may be used as an indicator of the onset of BPH deserves further scrutiny.

Keywords: Pyroglutamylglutamylprolineamide; Fertilization-promoting peptide; TRH-related peptide; Human prostate; Benign prostatic hyperplasia

1. Introduction

A peptide, pGlu-Glu-ProNH$_2$, which is structurally similar to TRH was first identified in the rabbit prostate complex [1] where concentrations increased markedly at sexual maturity and decreased with age [2]. The prostatic peptide is secreted in high levels into mammalian semen [3,4] pointing to a role in male fertility perhaps after ejaculation. In this context, nanomolar concentrations of pGlu-Glu-ProNH$_2$ have been shown, in vitro, to enhance capacitation of mouse epididymal spermatozoa with a concomitant increase in fertilizing ability [5]; for this reason the new peptide has been named fertilization-promoting peptide, using FPP as an acronym [5].

FPP has previously been detected in human semen [4], and, in this report, we investigate the nature of the TRH-related peptides in human prostate. High concentrations of FPP were detected in all samples of prostate, but in addition a second major peptide, distinct from TRH, and which was uncharged at all hydrogen ion concentrations, was always present in prostate from patients with benign prostatic hyperplasia (BPH). The possibility that this neutral TRH-related peptide is an indicator of BPH is discussed in this report. A portion of this work has been presented as a preliminary abstract [6].

2. Materials and methods

2.1. Materials

Synthetic FPP was obtained from Sigma Chemicals Ltd. (Poole, Dorset, UK), synthetic TRH was purchased from

Abbreviations: TRH, thyrotrophin-releasing hormone; FPP, fertilization-promoting peptide; BPH, benign prostatic hyperplasia

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Cambridge Research Biochemicals (Gadbrook Park, Northwich, Cheshire, UK) and ^{3}H-FPP from CRB-ICI (Billingham, Cleveland, UK). Sephadex resins were purchased from Pharmacia-LKB (Midsummer Boulevard, Central Milton Keynes, Bucks., UK). TRH antibodies were kind gifts from Dr Hamish Fraser (Edinburgh, UK) and Professor T.J. Visser (Rotterdam, The Netherlands).

2.2. Patients

BPH prostate

Tissue was removed by transurethral prostatectomy from six Caucasian men, aged between 60 and 76 years, with clinical indication of BPH: histological examination of the tissue at a later date confirmed the diagnosis (see Table 2). Tissue removed from each patient was frozen immediately in dry ice at −70°C.

Normal prostate

Prostatic tissue was removed from young Caucasian men aged between 32 and 46 (see Table 2). It was not possible to age-match patients from the two groups (BPH and Normal) because prostates from older men exhibit a degree of hyperplasia even in the absence of clinical symptoms. Microscopic examination of tissue sections stained with eosin/haematoxylin confirmed normal histology. For patients 8 and 9, tissue was removed both from the peripheral and transitional zones of the prostate. In three cases, tissue was obtained following cystoprostatectomy for bladder cancer which did not involve the prostate, and in two cases, prostate tissue was removed during open resection for urinary reconstruction. The tissue was frozen, immediately after the operation, in dry ice at −70°C.

Normal semen

After 3 days of abstinence, men presenting at the Assisted Conception Unit, University College Hospital, London provided semen samples for the study. Precisely 5 min after ejaculation, 1.0 ml semen was mixed with 5.0 ml acidified acetone (HCl/H_2O/acetone; 1:5:40, v/v) and stored at −20°C until analysis. Semen samples were used from men with normal sperm characteristics assessed using World Health Organisation directives; futhermore, subjects with known history of male infertility were excluded from the study. Samples for studies on peptide degradation were obtained from a donor bank of men at the Royal Veterinary College, London. Semen was collected at home by 6 subjects, and allowed to liquify for up to 2 h before the experiments were performed. A pooled sample was centrifuged at $3000 \times g$ (10 min) and the seminal plasma retained for analysis.

2.3. Rabbits

Male Sandy Lop rabbits were bred in a closed colony at the National Institute for Medical Research, London. At sexual maturity (4–5 months of age) the bucks were trained to mount a rabbit skin and ejaculate into an artificial vagina as described by Walton [7]. Semen collections were performed at weekly intervals. For analysis of TRH-related peptides, ejaculates of known volume were mixed with 5.0 ml of acidified acetone (as described above) and stored at −20°C until analysis. For estimations of peptide half-lives, freshly ejaculated rabbit semen samples from at least 3 animals were pooled, centrifuged ($3000 \times g$, 10 min) and the seminal plasma retained for the experiment which was performed within 30 min of ejaculation.

2.4. Extraction and identification of peptides

Detailed descriptions of extraction, separation and detection of TRH-related peptides have been described elsewhere [2]. To eliminate the possibility of cross-contamination, a blank was subjected to the entire procedure of extraction, separation and RIA before analysis of every sample. Prostate tissues were homogenized in ice-cold acidified acetone, and, after centrifugation, the supernatant dried in vacuo. Semen samples, already mixed with acidified acetone, were treated like the tissue but without the homogenization step. Dried extracts were reconstituted in 25% (v/v) acetic acid and the peptides desalted on a column (1×100 cm) of Sephadex G-10 eluting with 25% (v/v) acetic acid and collecting 1.0 ml fractions. Aliquots of each fraction were dried in vacuo and low molecular weight TRH immunoreactivity detected by radioimmunoassay (RIA) as described previously [2]. The TRH RIA will detect FPP, as well as authentic TRH, because the antibody used in this study has requirements for the N-terminal pyroglutamyl residue and C-terminal prolineamide but can accept substitutions at position 2; furthermore, the assay has the potential for detecting a variety of TRH-like peptides containing substitutions for histidine at position 2.

The chromatographic fractions (40–50) containing TRH-immunoreactivity were dried in vacuo and then the peptides dissolved in approximately 4.0 ml distilled water and adjusted to pH 7.6 with sodium hydroxide. TRH-related peptides were then resolved by anion-exchange chromatography on a column (0.7×60 cm) of QAE-Sephadex A25 Cl^- eluting with a linear gradient of NaCl (0–0.5 mol/l in 0.05 mol/l Tris-HCl pH 7.6; total volume 200 ml) and collecting 2.0 ml fractions [8,9]. Unbound TRH-immunoreactivity (fractions 7–9) was located by TRH RIA [2] and FPP (generally fractions 20–25) by RIA using an antibody which displays greater specificity for FPP than TRH itself [9]. Unbound TRH-immunoreactivity was further characterized by cation-exchange chromatography at pH 2.0 on a column (0.7×60 cm) of SP Sephadex C-25 Na^+ eluting with a linear gradient of NaCl (0–0.5 mol/l in 25% v/v acetic acid; total volume 200 ml) and collecting 2.0 ml fractions. TRH immunoreactivity was located in dried aliquots of fractions by RIA [2]. Recoveries of TRH

immunoreactivity after gel filtration chromatography were always greater than 90% with recoveries after anion-exchange chromatography of more than 80%.

3. Results

3.1. The stability of FPP in semen

As FPP appears to play a physiological role after ejaculation [5], it was important, in this study, to analyse the TRH-related peptides present in semen as well as prostate. In addition, we were interested in comparing TRH-related peptides in semen from normal men with those from men suffering from BPH; although it is proving difficult to obtain volunteers for the latter group. It was essential to establish a suitable protocol to ensure that peptides in semen remained intact until fixation in acidified acetone: this mixture is known to denature proteases and precipitate polypeptides with a molecular weight greater than 10 kDa and completely prevent the degradation of small peptides such as TRH [4]. In a series of experiments, the degradation of FPP and TRH was examined in both human serum, which is known to contain high concentrations of an enzyme that degrades TRH [10], and rabbit semen maintained at 37°C. The experiments were performed in the presence and absence of EDTA and DTT which inhibit the degradation of TRH in serum, and may have proved useful additives to collection tubes if they had also inhibited the degradation of FPP in semen. Rabbit semen was used for comparative studies because, unlike human semen, this secretion does not coagulate and can be used for experiments immediately after ejaculation.

TRH was degraded rapidly in untreated serum ($t_{1/2}$ = 12.5 min) but after addition of EDTA (ethylenediaminetetraacetic acid) and DTT (dithiothreitol) the rate of disappearance of peptide was markedly reduced (Fig. 1). In contrast, breakdown of FPP was undetectable in serum over a period of 2 h either in the presence or absence of EDTA and DTT (Fig. 1; up to 80 min illustrated). The degradation of both TRH and FPP varied markedly with the quality of the semen pool; for example, half-lives varied from 21–88 min (mean = 52.7, S.E. ± 19.9, $n = 7$) for TRH, and from 56–366 min (mean = 163.4, S.E. ± 51.3, $n = 6$) for FPP. In the presence of EDTA and DTT the degradation of both peptides in semen was accelerated (Fig. 1). The major effect was observed with FPP where degradation was accelerated nearly 4-fold ($t_{1/2}$ without EDTA + DTT = 301 min, $t_{1/2}$ with EDTA + DTT = 77 min). Incubation in the presence of EDTA and DTT also

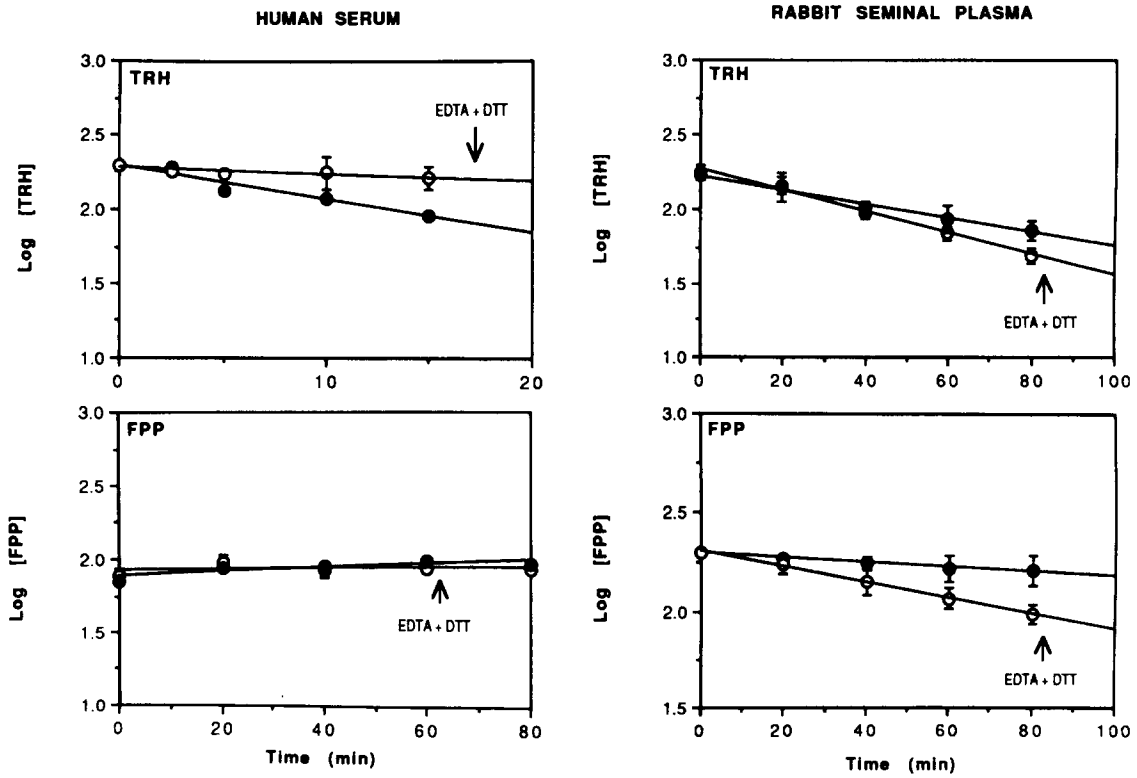

Fig. 1. The disappearance of TRH (top panels) and FPP (lower panels) after incubation with human serum (left panels) and rabbit seminal plasma (right panels). 100 pmol of TRH or FPP were added to 0.5 ml of serum or seminal plasma, in the presence (open circles) or absence (closed circles) of 2 mmol/l EDTA and 2 mmol/l DTT and incubated at 37°C for up to 2 h. At time intervals, aliquots were removed and mixed with an excess of acidified acetone, dried in vacuo and then subjected to RIA. Results represent mean (±S.E.) of 3 experiments for human serum and of 4 experiments for rabbit seminal plasma. The half lives of peptide disappearance were significantly different from controls after treatment with EDTA and DTT ($P < 0.05$; paired Student's t-test), with the exception of FPP degradation in serum.

decreased the half-life of disappearance of TRH but the effect was less pronounced than for FPP ($t_{1/2}$ without EDTA + DTT = 65.4 min, $t_{1/2}$ with EDTA + DTT = 46.3 min). Degradation of neither peptide could be detected, over a period of 2 h, in human semen which had been allowed to liquify after ejaculation.

3.2. Concentrations of FPP in normal human semen

Semen, classified as normal, was collected from 15 individuals as described in Section 2. The ejaculate was mixed with acidified acetone precisely 5 min after ejaculation, during which time insignificant degradation of TRH-related peptides occurred (Fig. 1). Analysis of the samples by anion-exchange chromatography (see Section 2) revealed only the presence of FPP with undetectable levels of neutral TRH-immunoreactive peptides (Fig. 2). Concentrations of FPP in each semen sample are shown in Table 1; mean FPP concentration was 49.4 nmol/l (S.E. ± 10.3). A similar range of FPP concentrations were observed in rabbit semen treated in an identical manner to the human samples; i.e., 14.4–151.0 nmol/l (mean = 51.2, S.E. ± 18.32, $n = 6$).

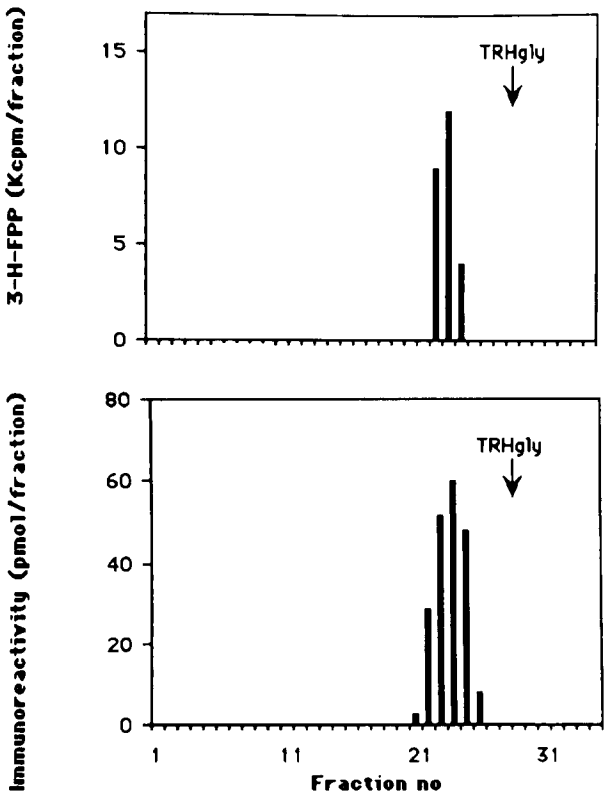

Fig. 2. TRH-related peptides present in normal human semen (lower panel) compared with the elution position of ^{3}H-FPP (top panel) after anion-exchange chromatography. Portions of each of the normal human semen samples collected into acidified acetone (see Table 1) were pooled, dried in vacuo and subjected to gel filtration, anion-exchange chromatography and RIAs for FPP and TRH, as described in Section 2. Radioiodinated TRHgly (1000 cpm) was used to indicate normal separation of peptides during chromatography.

3.3. TRH-related peptides in normal and BPH prostate

The TRH-immunoreactive peptides in prostate tissues were analysed by anion-exchange chromatography as described in Section 2. At pH 7.6, FPP will have an overall negative charge due to the ionization of the gamma-carboxyl moiety of the central glutamyl residue, and will bind to the positively-charged quarternary amino-ethyl functional group of the anion-exchanger. In contrast, TRH will have a partial positive charge at pH 7.6 and, along with TRH-like peptides with a neutral substitution at position 2, will elute straight from the column without binding to the resin. In this manner, FPP can be clearly resolved from TRH and neutral peptides present in the tissue extracts. Cation-exchange chromatography can then be used to resolve TRH from neutral peptides present in the unbound fractions after anion-exchange chromatography.

In prostate samples from all of the six patients with BPH, high concentrations of unbound immunoreactivity were observed in addition to FPP after anion-exchange chromatography (Fig. 3, Table 2). The majority of this unbound immunoreactivity consisted of a neutral tripeptide which co-eluted with FPP during gel-exclusion chromatography. However, low concentrations of TRH immunoreactivity co-eluting with synthetic TRH during cation-exchange chromatography at pH 2.0 were also present (< 10% of total TRH immunoreactivity; data not shown). It is possible that the neutral tripeptide is the predominant TRH-related peptide in BPH tissue but until its identity has been confirmed, the precise concentration cannot be assessed because of its unknown cross-reactivity with the TRH antibody.

In marked contrast, the major peptide present in normal prostate was FPP with low to undetectable levels of neutral TRH-like peptides (Fig. 3). Data from all patients is presented in Table 2. Unexpectedly, one sample (patient 9) in the 'normal' group showed significant levels of both peptides although this prostate may be anomalous: the patient is paraplegic and his prostate unusually small (approximately 3.0 g).

BPH involves hyperplasia of the peri-urethral (transitional) zone of the prostate and it is conceivable that the high levels of the neutral peptide observed in BPH tissue may also be associated with this region of the normal prostate. Peptides present in transitional and peripheral zones in 2 young patients, 8 and 9, were examined. Analysis of tissue extracts by anion-exchange chromatography revealed similar patterns of peptides in both zones (Table 2).

4. Discussion

The new prostatic peptide, pGlu-Glu-ProNH_2 (FPP), was previously purified and characterized from both rabbit prostate [1] and human semen [4]. The current study set out

to determine whether FPP was also present in human prostate, because it appears to be restricted to this tissue in the rabbit [2]. Human prostate is readily available from patients with BPH and initially the intention of the study was to examine the TRH-related peptides present in semen and prostate from patients with this condition. Firstly, a suitable protocol for the collection of semen samples was established by determining the half-lives of both TRH and FPP in semen samples incubated at 37°C.

Both peptides were degraded in rabbit semen although the rate of disappearance of TRH ($t_{1/2} = 53$ min) was generally more than twice that of FPP ($t_{1/2} = 163$ min). Interestingly, although TRH was degraded rapidly in human serum ($t_{1/2} = 12.5$ min), FPP remained intact during the 2 h incubation period, suggesting that the enzyme(s) which metabolises the new peptide may be different from the TRH-degrading enzyme in serum. Serum is known to contain high concentrations of pyroglutamyl aminopeptidase II (PAP II) which removes N-terminal pyroglutamic acid and is thought to be specific for TRH (for review, see [10]). The enzyme is a metalloproteinase and is inhibited by EDTA and DTT [11] which accounts for the slowing of degradation of TRH observed by addition of DTT and EDTA in this study (Fig. 1).

Our data indicate the presence of an enzyme in semen related to pyroglutamylaminopeptidase I (PAP I) which is generally thought to be located within the cytosol of the cell [10]. PAP I is a thiol protease and can remove the terminal pyroglutamyl residue from a number of unrelated short peptides; furthermore, enzyme activity is activated by EDTA and DTT [11] which may explain the acceleration of disappearance of both FPP and TRH incubated in rabbit semen in the presence of these two additives.

Due to the relatively slow degradation of TRH-related peptides, we were able to design a protocol whereby semen was mixed with acidified acetone precisely 5 min

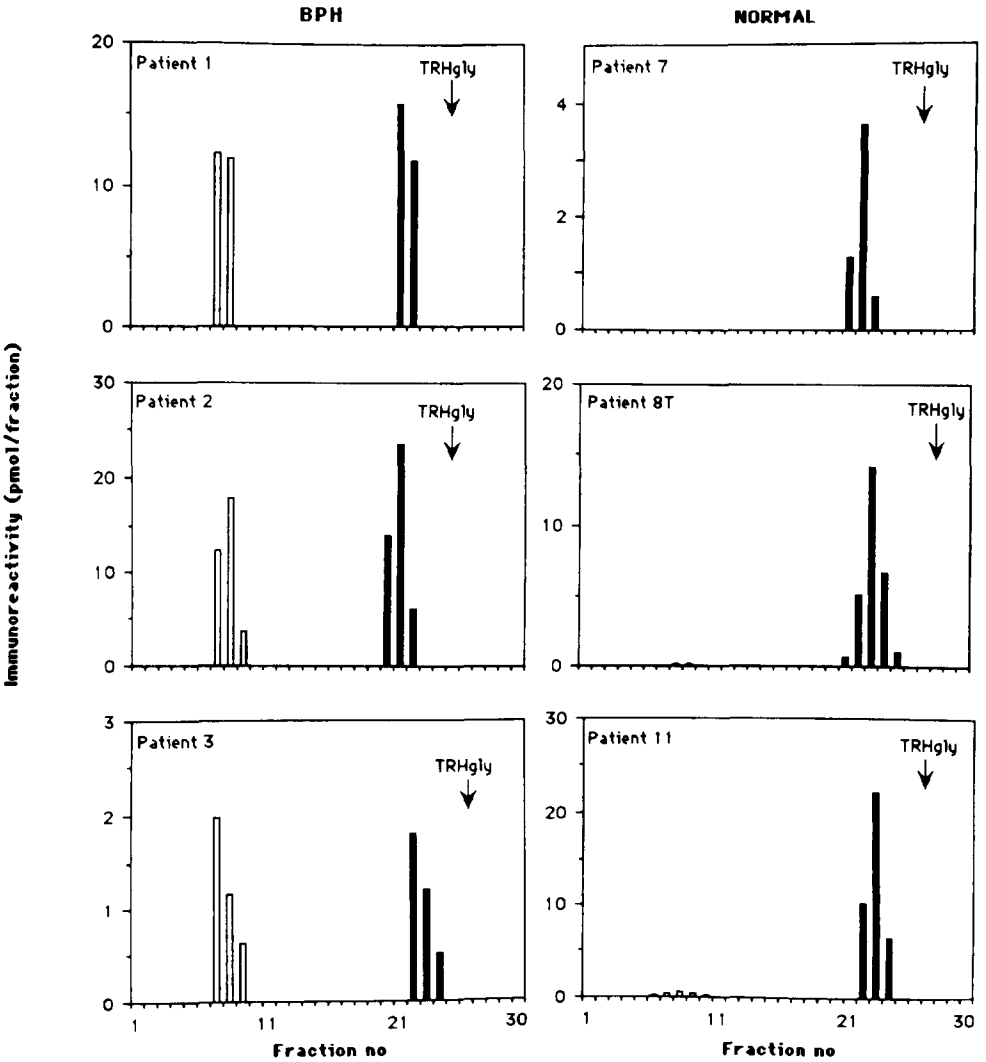

Fig. 3. TRH-related peptides present in normal (right panel) and BPH prostate (left panel) after anion-exchange chromatography. Peptides were extracted, desalted, subjected to anion-exchange chromatography and TRH (white blocks) and FPP RIAs (black blocks), as described in Section 2. Radioiodinated TRHgly (1000 cpm) was used to indicate normal separation of peptides during chromatography.

Table 1
Concentration of FPP in normal human semen

No.	Patient	Age	Children	Concentration (nmol/l)
1	322	30	No	61.5
2	321	33	No	29.5
3	338	31	Yes	40.0
4	330	43	No	27.0
5	283	31	No	47.0
6	254	29	No	18.0
7	151	38	Yes	41.5
8	299	40	No	50.3
9	270	31	No	48.5
10	268	29	No	73.5
11	233	39	Yes	181.5
12	290	41	Yes	27.0
13	236	28	No	17.5
14	237	41	Yes	48.0
15	272	37	No	30.5
				Mean 49.5
				S.E. ± 10.3

Semen samples were mixed with acidified acetone precisely 5 min. after ejaculation. Aliquots of the supernatant were dried in vacuo and then assayed for FPP as described previously [9].

after ejaculation. This time delay was sufficiently short to allow insignificant degradation of TRH-related peptides and, furthermore, abrogated the need for the patient to ejaculate directly into the fixative. Using this protocol, the average concentration of FPP in human semen was approximately 50 nmol/l which is also similar to that of rabbit semen (see Results). In this context, it is interesting that the effects of FPP on capacitation of mouse spermatozoa were observed above 10 nmol/l reaching maximal effectiveness between 50 and 100 nmol/l [5]: this suggests that the new peptide is present in semen in the range of concentrations (Table 1) that would be expected to elicit a physiological response in vivo.

Although analysis of TRH-related peptides in normal semen revealed only the presence of FPP (Fig. 2), a second TRH-immunoreactive peptide containing a neutral substitution at position 2 was observed in extracts of prostate from all BPH patients (Fig. 3). This neutral peptide appeared, after TRH RIA, to be present in similar amounts to FPP. In contrast to BPH tissue, the major peptide present in normal prostate was FPP, with barely detectable levels of the neutral tripeptide (Fig. 3). The identity of this neutral TRH-like peptide is unknown, but its presence in BPH tissue, in addition to FPP and TRH, has recently been observed by a second laboratory [12]. However, the occurrence of this new peptide does not appear to be restricted to hyperplastic tissue, because high levels were also observed in a young patient, without BPH, but with paralysis and associated impotency due to severe spinal damage (Table 2).

Table 2
Concentrations of neutral TRH-like peptide and FPP in normal and BPH prostate

Patient	Age	Tissue	Immunoreactivity (pmol/g)	
			Neutral	FPP
1	67	BPH	6.7	7.6
2	65	BPH	8.5	10.9
3	76	BPH	2.1	1.9
4	75	BPH	3.5	2.9
5	76	BPH	7.7	3.5
6	60	BPH	15.9	10.8
7	36	Normal: P	ND	1.60
8	46	Normal: P	0.2	4.1
		Normal: T	0.1	6.5
9	32	Normal: P	7.7	1.5
		Normal: T	7.4	0.9
10	46	Normal: P	ND	9.2
11	42	Normal: P	0.1	3.2

ND, not detectable; P, peripheral zone; T, transitional zone.

In summary, high levels of a neutral TRH-immunoreactive peptide are present in prostatic tissue from patients with BPH, but not from younger patients with normal prostate; thus, the expression of the neutral TRH-like peptide may be associated either with the onset of BPH or the ageing process. Although neutral TRH-related peptides are not normally detected in semen samples from young men [4] (Fig. 2), they have been detected in some samples of human semen, from individuals of unspecified ages [13,14], and recently, two such peptides, with Phe and Gln substitutions at position 2 of the tripeptide, have been characterized [14]: it is possible that one or both of these peptides may also be present in BPH prostate. Further work is required to examine the possibility that the presence of the neutral TRH-related peptide may be a useful indicator of the onset of BPH.

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