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Intranasal permeation of thyrotropin-releasing hormone: in vitro study of permeation and enzymatic degradation

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Abstract

The enzymatic degradation and permeation of thyrotropin-releasing hormone (TRH) were studied in vitro. No enzymatic degradation was observed in human nasal wash. The degradation rate in rabbit nasal mucosal homogenate and supernatant was about 0.5 μg/h (initial amount 1 μg), but it was possible to reduce the degradation rate by about 90% on the addition of 0.3% sodium glycocholate (GC). The apparent permeability coefficients of TRH over isolated rabbit nasal tissue mounted in the Ussing chamber were 4.94×10^{-6}, 12.63×10^{-6}, and 3.85×10^{-6} cm/s for an aqueous solution without enhancer, added 1% GC, and added 1% glycofurol 75 (GF), respectively. At the same time, the viability of the tissue in the Ussing chamber was studied by measurement of the electrophysiological properties. Addition of GC, but not GF, changed these properties significantly ($p < 0.01$).

Key words: Nasal; Ussing chamber; Permeation; Enzymatic degradation; Thyrotropin-releasing hormone

1. Introduction

Efficient biotechnological techniques and increasing knowledge about the physiological functions of peptides and proteins have made the need for alternative administration routes to parenteral routes more important. The most acceptable non-parenteral route of administration is the per oral route, however, it is not effective for peptides and proteins due to the presence of enzymes, poor absorption even though a peptide transport system for dipeptides and tripeptides is present (Friedman and Amidon, 1990), and hepatic first-pass metabolism. In contrast, the nasal route is one of those which has shown promising results for peptides and proteins, especially for smaller peptides (McMartin et al., 1987).

Thyrotropin-releasing hormone (TRH) is a tripeptide which is used clinically in the diagnosis of thyroid function. TRH is also expected to be of value in the treatment of various neurological and neuropsychiatric disorders (Metcalf and Jackson, 1989), such as schizophrenia and depression. Because of the low molecular mass of TRH (362 Da), TRH is believed to be rather well absorbed over the nasal mucosa (McMartin et al., 1987). Since TRH is rapidly metabolized in plasma, the half-life is about 9 min in vitro (Møss and Bundgaard, 1990), quantitative determina-

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tion of this compound is difficult in the systemic circulation. Consequently, the pharmacodynamic response of TRH has been used to estimate the bioavailability of TRH after intranasal administration in vivo (Sandow and Petri, 1985).

In particular, if the indications for TRH treatments are going to be extended, the need for alternatives to parenteral administration of TRH will grow. Knowledge about the stability and absorption of TRH by different administration routes will be of value in an evaluation of the different alternative routes.

The aim of this study was to estimate the stability of TRH against nasal enzymes and to examine the possibility of enhancing the nasal permeation of TRH by use of enhancers. Further, the results are a part of an evaluation of the Ussing chamber method.

2. Materials and methods

2.1. Chemicals

Thyrotropin-releasing hormone (TRH) was kindly provided by Carlbiotech A/S, Copenhagen, Denmark. Glucose was obtained from May and Baker (Dagenham, U.K.). Sigma 7–9® Biochemical buffer Tris (99.0–99.5%), sodium glycocholate (approx. 99%) and bovine serum albumin (RIA grade) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glycofurol 75 was obtained from Roche (Basle, Switzerland). ^{14}C-labelled polyethylene glycol 4000 (PEG-4000) is commercially available from New England Nuclear (Du Pont, Boston, MA, U.S.A.). Acetonitrile of HPLC grade was purchased from Romil Chemicals (Leicester, U.K.). Carbogen (95% O_2/5% CO_2) was obtained from Hede Nielsen A/S (Denmark). Scintillation cocktail (Pico-aqua) was obtained from Packard Instrument BV (Groningen, The Netherlands). All other chemicals were of analytical grade.

2.2. Apparatus

The acrylic Ussing chamber with accessories and scintillation counter were the same as described previously (Bechgaard et al., 1992).

2.3. Analysis

The Hitachi HPLC system, used for quantitative analysis of TRH, consisted of a L-6000 pump, a L-4000 variable-wavelength UV detector and a Rheodyne® 7125 injection valve (Berkeley, CA, U.S.A.), equipped with a 20 μl loop. The column was a 4 × 250 mm LiChrosorb® RP-18 (5 μm) and the guard column was a LiChroCART® 4–4 from Merck (Darmstadt, Germany).

The mobile phase was a mixture of solution A and water (1:7 or 1:9). Solution A (pH 2.5) consisted of 0.04 M phosphoric acid, 0.2 M sodium sulphate and 10% acetonitrile. The conditions used were: detection, at 214 nm; flow rate, 1 ml/min; column temperature, 25°C and injection volume, 20 μl. The retention time was about 8–11 min. Sample concentrations were calculated on the basis of peak height relative to external TRH standards.

The detection limit was 0.1 μg/ml and the precision 8%.

Samples from the mucosal side were diluted 1:79 with mobile phase.

2.4. Tissue preparation

Rabbit nasal mucosal tissue from the septum (Gizurarson and Bechgaard, 1991) was obtained from New Zealand White rabbits (about 3 kg) kindly provided by Novo Nordisk A/S (Denmark). The tissue was immediately placed in oxygenated glucose bicarbonate-Ringer solution (GR, Bechgaard et al., 1992) or homogenized.

2.5. Isolation of nasal enzymes

Rabbit nasal homogenate and supernatant enzyme preparations were prepared from about 100 mg freshly removed rabbit nasal mucosal tissue (Gizurarson and Bechgaard, 1991). Human nasal wash was collected after instillation of 2 ml of isotonic saline solution in each nasal cavity and used immediately after (Gizurarson and Bechgaard, 1991).

Protein content was determined as described by Miller (1959) with slight few modification.

2.6. Enzymatic degradation study

The study was conducted in glass vials (Maple Leaf Brand 12 × 75 mm, MacMillan Bathurst Inc., Canada) at 37°C. 1 ml 0.1 M Tris buffer and 0.5 ml enzyme preparation were mixed and preheated to 37°C. The reaction was initiated by adding 1 ml of 1 μg TRH/ml 0.1 M Tris buffer. At different times during a 1 h period, 25-μl samples were withdrawn and immediately analysed by HPLC.

2.7. Permeation study

The tissue preparation and Ussing chamber set up were as described previously (Bechgaard et al., 1992). The tissue was preincubated 60 min for stabilisation. GR and 0.3 w/v% albumin in GR (GRA) were added to the mucosal and serosal side, respectively. In this period and during the permeation study the transepithelial potential difference (PD) and the short-circuit current (I_{sc}) were measured. After preincubation 500 μl GR was replaced with a solution containing 400 μg TRH/ml, resulting in a final concentration of 200 μg TRH/ml at the mucosal side. GC and GF were added at both sides after 55 min of preincubation in the respective studies. At different times during a 90 min period, samples for analysis of TRH were withdrawn from the mucosal and serosal side. The sample volumes were replaced.

2.8. Disappearance study

To determine the possible disappearance rate of TRH from the serosal side, 1 μg TRH was added to the serosal side immediately after the 90 min permeation study. Samples were withdrawn three times during a 39 min period and analysed immediately. After the disappearance study the tissue integrity was examined with PEG-4000 as described by Bechgaard et al. (1992).

2.9. Calculation

The degradation rate (according to the enzymatic degradation study) is equal to $-\alpha \times 60$ min/h, where α is the slope of the regression line.

The concentrations of TRH during the permeation study and disappearance study were corrected for dilution using the following equation:

$$Q = V_s\left(\sum_{n=1}^{n} C_{n-1}\right) + C_n V_t$$

where Q is the total amount of TRH, V_s denotes the sample volume, V_t is the chamber volume and $C_{1,2,\ldots,n}$ represents the concentration of sample $1,2,\ldots..,n$.

The concentration at 90 min is assumed to be equal to the recovery at 85 min + $\alpha \times 5$ min.

The appearance rate is equal to $\alpha \times 60$ min/h, where α is the slope of the regression line using the values from 15 to 85 min.

The correction for degradation and/or adsorption is evaluated from the following equation:

$$\text{Corr}\ (\%/\text{h}) = (B-C)\frac{100}{1/2(A+B)} \times \frac{60}{t_b - t_a}$$

where A and B are the theoretical values at 90 and 129 min, respectively. A is equal to the recovery at 85 min + $\alpha \times 5 + 1$ μg and $B = A + \alpha \times 39$. C is the measured value at 129 min corrected for dilution. t_a and t_b are the times corresponding to A and B, respectively.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{CA}(\text{cm/s})$$

where dQ/dt is the slope of the regression line, C denotes the concentration at the mucosal side and A is the surface area (0.5 cm^2) of the exposed nasal mucosal tissue.

3. Results and discussion

3.1. Enzymatic degradation study

The degradation rates of TRH were found to follow first-order kinetics (Fig. 1) and are listed in

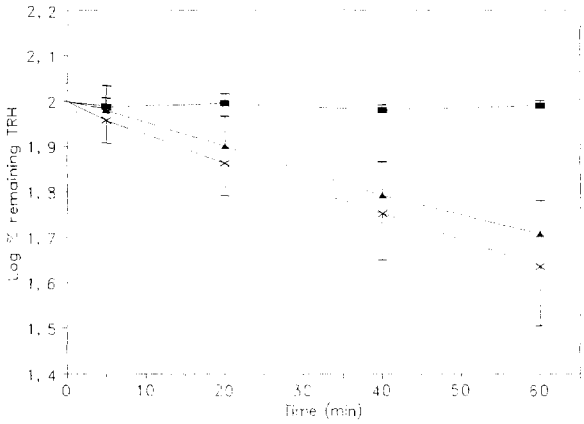

Fig. 1. First-order plots (mean ± S.D.) for the degradation of TRH in an aqueous solution (■), in rabbit nasal homogenate (×), and in rabbit nasal supernatant (▲) at 37°C. Initial amount of TRH was 1 μg.

Table 1. Human nasal wash did not affect the degradation rate, whereas rabbit nasal homogenate and supernatant increased the rate of degradation substantially. The degradation rate was 0.499 μg/h (49.9%/h) in rabbit nasal homogenate and 0.488 μg/h (48.8%/h) in rabbit nasal supernatant compared to 0.012 μg/h (1.2%/h) in the control experiments, i.e., the degradation rate was increased by a factor of 40 after addition of the rabbit nasal enzyme preparations.

Addition of even very small amounts of GC inhibited the enzymatic degradation substantially. It does not appear to be possible to inhibit the enzymatic degradation of TRH with GC completely, since no decrease in degradation rate is observed when increasing the amount of GC from 0.3 to 1.0%. The inhibitory effect of GC on enzymatic degradation has also been demonstrated with other peptides (Hirai et al., 1981; Igawa et al., 1989; Faraj et al., 1990; Yamamoto et al., 1990). Generally, it has been possible nearly to abolish the enzymatic degradation of the peptides (Hirai et al., 1981; Igawa et al., 1989; Faraj et al., 1990), consistent with the observation in this study.

A solution of TRH administered intranasally is expected to be reasonably stabile in the nasal cavity, as no enzymatic degradation is observed in human nasal wash, and addition of an enzyme inhibitor seems unnecessary. However, depending on the maximal enzymatic degradation rate by human intracellular nasal enzymes and the absorption route (transcellular or paracellular), addition of an enzyme inhibitor may be of clinical value.

As the degradation of TRH is determined in rabbit nasal homogenate and supernatant (TRH is exposed to both intracellular and extracellular enzymes), it is probably overestimated relative to the degradation of TRH occurring during the

Table 1
Influence of human nasal wash, rabbit nasal homogenate ± addition of glycocholate (GC), and supernatant on the degradation rate of TRH (initial amount 1.0 μg) (all values are expressed as mean ± S.D.)

Experiment	n [a]	Degradation rate (μg/h)	Protein content (mg/ml)	t-test [b]
Human nasal wash	6	0.058 ± 0.101	0.345 ± 0.060	$p > 0.05$
Control	6	0.011 ± 0.016	–	–
Homogenate	6	0.499 ± 0.057	2.873 ± 0.902	$p < 0.001$
Supernatant	6	0.488 ± 0.055	1.483 ± 0.120	$p < 0.001$
Control	6	0.012 ± 0.016	–	–
Homogenate + 0.1% GC	1	0.223	2.815	–
Homogenate + 0.2% GC	1	0.105	2.443	–
Homogenate + 0.3% GC	3	0.049 ± 0.015	3.713 ± 0.721	$p < 0.05$
Homogenate + 1.0% GC	1	0.047	[c]	–

[a] Number of observations.
[b] Degradation rate tested by two-sample t-test.
[c] Not measured.

Table 2
TRH at serosal side in % (mean ± S.D.) of the initial amount at mucosal side (200 μg) at different times during the experiment

Time (min)	Control ($n = 6$)	+1% glycocholate ($n = 6$)	+1% glycofurol 75 ($n = 4$)
15	0.18 ± 0.15	0.29 ± 0.19	0.17 ± 0.03 [b]
35	0.46 ± 0.32	1.05 ± 0.59 [a]	0.39 ± 0.20
55	0.75 ± 0.46	1.70 ± 0.89	0.63 ± 0.29
70	0.96 ± 0.59	2.31 ± 1.22	0.78 ± 0.39
85	1.23 ± 0.64	3.20 ± 1.50 [a]	0.95 ± 0.46
101	1.90 ± 0.75	4.26 ± 1.78	1.82 ± 0.50 [c]
115	2.10 ± 0.81	5.03 ± 2.05	1.84 ± 0.63
129	2.36 ± 1.09	5.79 ± 2.26	1.96 ± 0.68

At 90 min 0.5% (1 μg) TRH was added to the serosal side to estimate the correction for degradation.
[a] $n = 5$; [b] $n = 2$; [c] $n = 3$.

permeation study, depending of its pathway of movement through the tissue.

3.2. Permeation and disappearance study

The amount of TRH (mean) permeating through the isolated rabbit nasal tissue during a period of 85 min was determined to be 1.23% from an aqueous solution (Table 2). The corresponding value after addition of 1% GC or GF is 3.20 and 0.95%, respectively.

As seen from Table 3, 1% GC enhanced the appearance rate significantly ($p < 0.001$), whereas 1% GF did not change the rate of appearance. The mechanism by which bile salts enhance the permeation of drugs through mucous membranes appears to be due to a combination of effects. Some of the mechanisms of GC are known. It reduces the viscosity and elasticity of mucus (Martin et al., 1978), inhibits proteolytic enzymes (Hirai et al., 1981), and alters membrane structure (Hirai et al., 1981). As the correction for degradation and/or adsorption in the calculation of appearance rate is insignificant (0.4–6.7%), the enhancement of TRH permeation by addition of GC is probably not due to the inhibitory effect of GC on enzymatic degradation. The inhibitory effect cannot be completely excluded as the disappearance study only shows what disappears at the serosal side and not what happens to TRH when it moves through the tissue. The presence of mucus at the isolated nasal tissue is expected to be minimal because of washing under and after isolation, therefore the observed permeation enhancing effect of GC is probably mainly due to alteration of membrane integrity.

GF has been shown in vivo to enhance the nasal bioavailability of insulin in rabbits significantly (Bechgaard et al., 1991), however, in vitro it has not been possible to demonstrate any effect of GF on the permeation of peptides and mannitol (Jørgensen and Bechgaard, 1993; Jørgensen et al., 1993), neither has GF an effect on the permeation of TRH. The different observations with GF could be due to the fact that the mechanism by which GF enhances permeation is only detectable in vivo (e.g., influence on the viscosity of mucus or mucociliary clearance) or is simply due to a difference in the nature of the peptides.

The apparent permeability coefficients for TRH through different tissues have been determined in vitro. The absorption of TRH through isolated intestinal segments from rats mounted in the Ussing chamber resulted in P_{app} values of 8.9×10^{-6}, 7.4×10^{-6}, and 5.8×10^{-6} cm/s for

Table 3
Influence of sodium glycocholate (GC) and glycofurol 75 (GF) on appearance rate, corrected appearance rate, and apparent permeability coefficient for TRH

Experiment	Appearance rate (μg/h)	Corrected appearance rate [a] (μg/h)	P_{app} ($\times 10^6$) (cm/s)	t-test [b]
Control	1.78 ± 0.88	1.90 ± 0.86	4.94 ± 2.43	–
1% GC	4.54 ± 2.24	4.56 ± 2.23	12.63 ± 6.22	$p < 0.001$
1% GF	1.39 ± 0.71	1.44 ± 0.76	3.85 ± 1.98	$p > 0.05$

Experiments were conducted in the Ussing chamber mounted with rabbit nasal tissue. All values are expressed as mean ± S.D.
[a] When Corr (%/h) is negative, the appearance rate is assumed to be equal to the corrected appearance rate.
[b] Appearance rate tested by two-sample t-test.

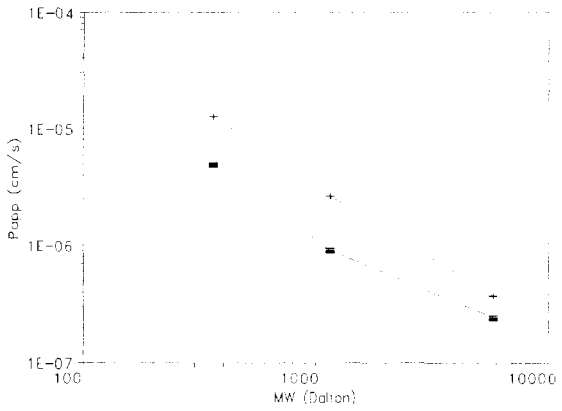

Fig. 2. Relationship between molecular weight (MW) and apparent permeability coefficient (P_{app}) for peptides (TRH, angiopeptin, and insulin) studied in the Ussing chamber. (■) Studies without addition of enhancers; (+) studies after addition of enhancers (1% GC for the two lowest MW and 2% didecanoylphosphatidylcholine for the highest MW).

jejunum, ileum, and colon, respectively (Møss et al., 1990). P_{app} for TRH over Caco-2 cell monolayers was about 1.5×10^{-6} cm/s (Lundin et al., 1991) and over rabbit buccal mucosa about 0.14×10^{-6} cm/s (Dowty et al., 1992). The observed P_{app} for TRH over rabbit nasal tissue is about 3-fold greater than over Caco-2 cell monolayers and more than 35-fold higher than over buccal tissue, whereas the permeation over rat intestinal tissue is slightly greater than over rabbit nasal tissue.

As seen from Fig. 2 (including previous results; Bechgaard et al., 1992, 1993; Jørgensen and Bechgaard, 1993), a linear relationship between log molecular weight and log P_{app} for peptides over rabbit nasal mucosa studied in vitro was obtained. This also seems to be true when enhancers are added. This relationship, however, must be investigated further with other peptides and enhancers.

The bioavailability of TRH in rats has been estimated to be about 20% (Sandow and Petri, 1985), i.e., about 20-fold more than the amount of TRH permeating the rabbit nasal mucosa in 85 min in vitro (Table 2). In addition to the difference in animal species, the absence of blood flow in the subepithelial tissue in the in vitro system may be the reason for the large difference in bioavailability.

Table 4 shows the electrophysiological properties of the isolated rabbit nasal tissue before and after the permeation study. There is no statistically significant difference ($p > 0.05$) in the initial I_{sc} and PD in the experiments except for the PD in the experiments with 1% GC. No differences ($p > 0.05$) in the electrophysiological properties of the rabbit nasal tissue before and after the penetration study were seen in the experiments with no enhancer and 1% GF, whereas 1% GC changed the electrophysiological properties significantly ($p < 0.01$), indicating a toxicological effect of GC, but not of GF, on the tissue at the concentration used.

The tissue resistance of rabbit nasal tissue was about 50 Ω cm^2 compared with about 250 Ω cm^2 in cultured monolayers of Caco-2 cells (Artursson, 1990) and about 2000 Ω cm^2 in porcine buccal mucosa (Hansen et al., 1992). This indicates a more leaky nature of the nasal mucosa compared with the other two mucosae, and is consistent with the observed differences in P_{app} values.

Table 4
Short-circuit current (μA/cm^2) and potential difference (mV) across rabbit nasal tissue mounted in the Ussing chamber

Experiment	After preincubation		After penetration study	
	μA/cm^2	mV	μA/cm^2	mV
Control	70.0 ± 26.7 [a]	2.5 ± 1.2 [a]	73.0 ± 24.0	2.4 ± 1.3
1% glycocholate	49.2 ± 30.5 [b]	0.9 ± 0.8 [b]	5.0 ± 8.9	0.1 ± 0.2
1% glycofurol	35.5 ± 18.4 [b]	2.5 ± 2.6 [b]	42.0 ± 12.0	2.4 ± 0.7

All values are expressed as mean ± S.D.
[a] After 60 min of preincubation.
[b] After 55 min of preincubation.

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