J.Pharm.Dyn., 7, 445–451 (1984)

INTESTINAL ABSORPTION MECHANISMS OF THYROTROPIN-RELEASING HORMONE

SHIGEHARA YOKOHAMA, TOSHIO YOSHIOKA, KENJI YAMASHITA AND NOBUYUKI KITAMORI

Central Research Division, Takeda Chemical Industries, Ltd., Juso-honmachi, Yodogawa-ku, Osaka, 532, Japan

(Received November 21, 1983)

Intestinal absorption mechanisms of thyrotropin-releasing hormone (TRH) following the oral administration of TRH-tartrate (TRH-T) were studied in animals. When TRH-T was orally administered to rats or beagle-dogs, absorption of TRH showed apparent saturation and decreased with food ingestion. TRH is very stable against gastrointestinal digestive enzymes, homogenized intestine and epithelial cells. First pass effect in the liver was not observed in beagle-dogs. Absorption site specificity was found in rats, namely TRH can be absorbed from only the upper part of the small intestine. A saturation phenomenon was also observed in *in situ* and everted sac experiments. TRH absorption was inhibited by the existence of oligopeptides and some β-lactam antibiotics that had been reported to be absorbed by active transport or carrier-mediated transport systems. The transfer of TRH from mucosal to serosal solutions was inhibited by the replacement of medium Na ions by K ions and by the existence of oligopeptides. The transfer rate from serosal side to mucosal side was much slower than that from mucosal side to serosal side. These results suggested that there should be a certain carrier-mediated transport system in the absorption process of TRH.

Keywords—thyrotropin-releasing hormone; thyrotropin-releasing hormone tartrate; radioimmunoassay; intestinal absorption; carrier-mediated transport; absorption site

INTRODUCTION

Since orally administered thyrotropin-releasing hormone (TRH) has been reported to enhance thyroid stimulating hormone release in man,[1] it was predicted that TRH should be absorbed from gastrointestinal tract. It was confirmed by a specific radioimmunoassay that TRH was absorbed after oral administration of thyrotropin-releasing hormone tartrate (TRH-T) to rats, dogs, and humans.[2,3] This absorption, however, showed apparent saturation and was inhibited by food ingestion. In a series of papers by Agar, Wiggans, Craft and Adibi they reported that oligopeptides and amino acids were absorbed by specific carrier-mediated systems. Both systems, however, were not identical and did not interfere with each other.[4-7] Addison reported that absorption of di- and tripeptides competed with each other but that of tetrapeptides did not.[8] Matthews also described in his reviews that a possible mechanism of absorption of some biologically active peptides may be diffusion through aqueous pores, and a failure to meet the structural requirement for carrier-mediated peptide transport by the small intestine and relatively large molecular volume (molecular weight 362) may explain why the absorption of thyrotropin-releasing factor, pyroglu-his-pro-NH_2 is so slight; for though the size of aqueous pores may cover a wide range, it is unlikely that molecules of over 200 molecular weight can be absorbed through such pores on any substantial scale.[9] However, if the absorption of TRH is occurred by diffusion through aqueous pores and does not compete with that of oligopeptides, the phenomena of apparent saturation and inhibition with food ingestion can not be adequately explained. In this paper we report on the absorption of TRH across the small intestine *in situ* and *in vitro* designed to elucidate the transport mechanism involved.

MATERIALS AND METHODS

Animals —Experimental animals used in this study were described proviously.[2,3]

Preparation of Drug Solution —TRH-T was dissolved in isotonic buffer solution of NaH_2PO_4-Na_2HPO_4, pH 6.5.

Materials —The following reagents were used: TRH-T (L-pyroglutamyl-L-histidyl-L-prolinamide-L-tartrate monohydrate, Takeda), ^{125}I-TRH (specific activity: 140 μCi/μg), anti-TRH serum, goat anti-rabbit γ-globulin serum, and normal rabbit serum were prepared as described previously.[2] Pepsin, trypsin, and chymotrypsin were purchased from Wako Pure Chem., and all other materials and solvents were of analytical reagent grade and used without further purification.

Radioimmunoassay —Radioimmunoassay of TRH was performed by the procedure described previously.[2]

Incubation with Gastrointestinal Digestive Enzymes, Homogenized Intestine and Epithelial Cells —TRH solutions were incubated with 30 mg of pepsin, 30 mg of trypsin, or 5 mg of chymotrypsin at 37°C for 60 min. After the incubation, the samples were boiled at 100°C for 1 min to terminate reactions. Thin-layer chromatography (TLC) analysis was carried out on a silica gel plate as described by Nakajima.[10] Rats were injected intraperitoneally with 100 mg/kg of sodium phenobarbital and 50 mg/kg of sodium pentobarbital to induce anesthesia and the intestine (after washed with pH 6.5 isotonic sodium phosphate buffer solution) were removed, and homogenized with Tyrode's solution. Peptide contents of the samples were determined by the use of the folin reagent,[11] and diluted to 5 mg/ml peptide contents. TRH was incubated with the homogenized solution at 37°C and the residues were assayed by the radioimmunoassay of TRH.

First Pass Effect in the Liver —Beagle-dogs were anesthesized with 30 mg/kg of sodium pentobarbital and their abdomens incised. After TRH-T solution (*eq.* 100 μg/kg of TRH) was injected into the portal or antecubital vein, 1.5 ml of heparinized venous blood samples were collected from the other site of the antecubital vein periodically and quickly poured into 7 ml of methanol (MeOH). After the extraction and the evaporation under dried N_2 gas, the radioimmunoassay of TRH was performed.[2]

In Situ Absorption Studies in Rats —Rats were injected intraperitoneally with 100 mg/kg of sodium phenobarbital and 50 mg/kg of sodium pentobarbital to induce anesthesia and *in situ* absorption studies similar to those described by Noguchi were carried out.[12] The bile duct was ligated. One milliliter of the drug solution was injected into the loop of the upper part (10 cm length from the pylous), the middle part (10 cm), or the lower part (10 cm) of the small intestine. One hundred microliters of arterial blood samples were collected from the jugular artery periodically and quickly poured into 2—4 ml of MeOH. After the extraction and evaporation under dried N_2 gas, the radioimmunoassay of TRH was carried out.

Permeation through the Small Intestine —The everted sac (10 cm) from the upper, middle, or lower part of rat small intestine was prepared according to the method of Wilson and Wiseman.[13] The everted sac was placed in a 50 ml flask with 30 ml of modified Ringer's solution.[13] The flask was gassed with the mixture composed of 95% O_2 and 5% N_2 maintaining at 37°C. The serosal initial volume was 1.0 ml. At the end of the incubation period (normally 30 min), 100—200 μl from sac was poured into 2 ml of MeOH. After the extraction and evaporation by a stream of dried N_2 gas, the radioimmunoassay of TRH was carried out (if necessary, after diluting).

RESULTS AND DISCUSSION

The blood concentration curves of TRH after intra-antecubital or intra-portal vein injection were almost identical in beagle-dogs (Fig. 1), and the same observation was obtained in rats. Therefore, the liver is not a principal degrading site and the first pass effect can not explain the insufficient absorption of TRH-T.

The stability of TRH-T against gastrointestinal digestive enzymes, homogenized rat intestine and

epithelial cells are summarized in Table I and Fig. 2. TRH-T is very stable on the route of absorption from the small intestine against the gastrointestinal digestive enzymes. Sometimes polypeptides, for example insulin, are degraded by gastrointestinal digestive enzymes,[14] furthermore dipeptides and tripeptides are reported to be adequately absorbed from the small intestine with some degradation to liberated amino acids.[9] It is suggested that the poor availability of levodopa resulted from its metabolism in the intestine during absorption.[15] However, the specific phenomenon of the absorption of TRH can not be explained by hydrolysis in the intestine or liver.

Absorption site specificity of TRH was studied by the *in situ* method. Doses were 0.06, 0.29, and 1.46 mg/kg of TRH-T (*eq.* 0.04, 0.2, and 1.0 mg of TRH). It was found that TRH was absorbed mainly from the upper small intestine, and little or none was absorbed from the middle or lower small intestine. Moreover, an apparent saturation phenomenon of the absorption from the upper small intestine was observed (Fig. 3). Di- and tripeptides were reported to be absorbed from some specific range of the gastrointestinal tract, but this does not mean some specific ranges of the small intestine;[16] di- and tri-peptides can be absorbed from all parts of the small intestine even if the absorption rate is slightly different in the different parts.

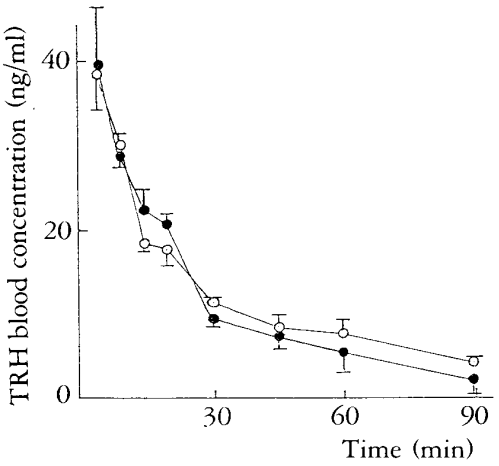

FIG. 1. *Blood Concentration of TRH after Intra-antecubital or Intra-portal Vein Injection in Beagle-dogs*

Dose: 100 μg/dog; ○, intra-antecubital vein; ●, intra-portal vein. Data point represents the average of three experiments: the vertical bar represents the standard error of the mean.

In our previous paper,[3] we reported that the bioavailability of TRH after food ingestion was about half of that in the fasting state, and this phenomenon was presumably caused by some contents of food. Addison reported that absorption of dipeptides was inhibited by another peptides.[8] Therefore, in oder to ascertain the absorption mechanisms, the relation between the absorption systems for TRH and those of oligopeptides and aminoacids, known to be transported by a specific carrier-mediated system, was investigated. Fig. 4 shows the absorption of TRH when TRH-T was injected into the lumen of the ligated loop of the rat upper small intestine with or without casaminoacids (aminoacids mixture) and oligopeptides mixture. Absorption was inhibited by the oligopeptides mixture but not by casaminoacids. This observation suggests that the absorption of TRH shares a common carrier system with oligopeptides but not with aminoacids.

TABLE I. *Stability of TRH to Gastrointestinal Digestive Enzymes*

Peptidase	TRH-T (mg)	Medium	Incubation	Stability (TLC)
Pepsine 30 mg	73	pH1.2 HCl buf.	37°C 60 min	One spot
Trypsine 30 mg	73	pH7.5 Phos. buf.	37°C 60 min	One spot
Chymotrypsine 5 mg	73	pH7.5 Phos. buf.	37°C 60 min	One spot

Pepsin, Wako (1:10 000); trypsin, Wako (2 000 U/g); chymotrypsin, ICN Pharmaceuticals (45 U/mg).

It has been reported that the absorption of some β-lactam antibiotics, which were considered to be one of the oligopeptides, are inhibited by oligopeptides;[8,17] thus observation have revealed the existence of the active transport for cyclacillin and cephalexin, of the facilitated diffusion mechanism for amoxicillin, and no evidence of carrier-mediated transport of ampicillin. Then, the inhibition against absorption of TRH by those antibiotics was studied. The dose of TRH-T was 292 μg/kg (*eq.* 200 μg of TRH) and antibiotics were 2 000 μg/kg. The results are shown in Table II. While amoxicillin, cyclacillin, and cephalexin inhibit the absorption of TRH, ampicillin does not. These data agree with the fact that cyclacillin, amoxicillin, and cephalexin are absorbed by some carrier-mediated systems, shared with oligopeptides or facilitated diffusion mechanisms, whereas ampicillin has very poor affinity to the systems.

In order to clarify the contribution of carrier systems to the absorption processes of TRH and the absorption site specificity, everted sac experiments were carried out. Table III shows the effect of some β-lactam antibiotics on the permeation of

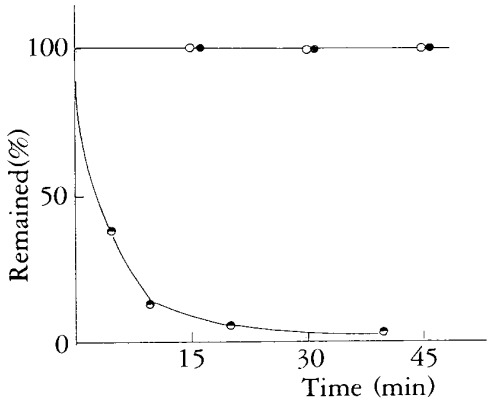

FIG. 2. *Disappearance of TRH during Incubation at 37°C in Vitro (Initial TRH-T Concentration: 14.6 ng/ml)*

○, intestine of rats; ●, epithelial cells of rats; ◓, blood of rats.

Each point represents the average of three experiments.

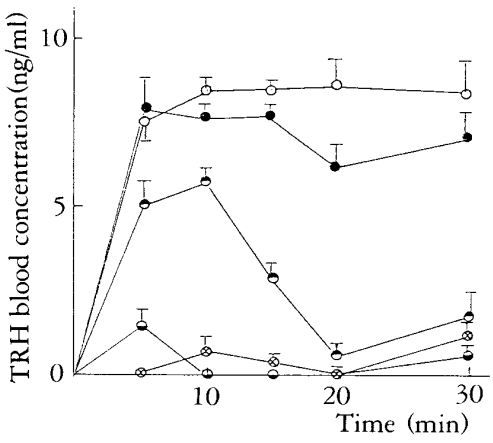

FIG. 3. *TRH Blood Concentration Curve on in Situ Absorption Experiments from Rat Small Intestinal Loop*

●, upper part (dose: TRH-T 1.46 mg/kg); ○, upper part (0.29 mg/kg); ◓, upper part (0.06 mg/kg); ⊗, middle part (0.29 mg/kg); ◒, lower part (0.29 mg/kg).

Data point represents the average of four experiments: the vertical bar represents the standard error of the mean.

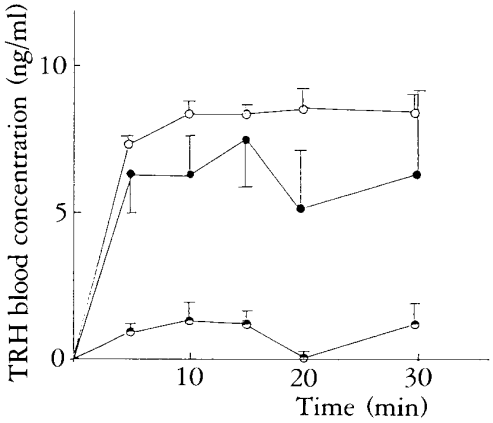

FIG. 4. *Effect of Oligopeptides Mixture and Casaminoacids on the in Situ Absorption Experiments from Rat Intestinal Loop*

○, with no additives; ●, with casaminoacids (10 mg/kg); ◓, with oligopeptides mixture (gly-gly 10 mg/kg + β-ala-his 10 mg/kg + gly-gly-gly 10 mg/kg + gly-pro 10 mg/kg).

Data point represents the average of four experiments: the vertical bar represents the standard error of the mean.

TABLE II. *Effect of β-Lactam Antibiotics on the Absorption of TRH from Rat Small Intestine in Situ Experiments*

β-Lactum antibiotics	*AUC* ng·h/ml	%inhibit	Statistical significance (*p*)[a]
None	38.5	—	—
Cyclacillin	6.9	82.1	$p < 0.01$
Amoxicillin	4.1	89.3	$p < 0.01$
Ampicillin	40.9	0	N.S.
Cephalexin	25.2	34.5	$p < 0.10$

a) Student's t-test, N.S. = not significant, n = 4–6.

TABLE III. *Effects of β-Lactam Antibiotics on the Permeation of TRH from Mucosal to Serosal Fluids in Everted Sac Experiments*

β-Lactum antibiotics	Absorbed (ng)	Statistical significance (*p*)[a]
None	355	—
Cyclacillin	112	$p < 0.05$
Amoxicillin	150	$p < 0.05$
Ampicillin	382	N.S.
Cephalexicin	168	$p < 0.05$

a) Student's t-test, N.S. = not significant, n = 4–6.
(Initial TRH-T concentration is 14.6 ng/ml and those of antibiotics are 100 ng/ml).

TRH. The concentration of TRH-T in a mucosal side is 14.6 ng/ml (*eq.* 10 ng of TRH) and the concentration of antibiotics is 100 ng/ml. Cephalexin, amoxicillin, and cyclacillin inhibited the permeation of TRH across the everted sac, and ampicillin did not. These results coincide with those of *in situ* experiments. The relation between the degrees of the inhibition by the antibiotics in *in vitro* and *in situ* experiments was not apparent.

In order to ascertain the existence of a kind of active transport systems for the absorption of TRH, the effect of the replacement of Na ions by K ions, the effect of existence of oligopeptides on the permeation of TRH, and non-everted/everted sac transporting rate ratio of TRH were examined. Fig. 5 shows the results. Permeation of the drug was inhibited by the existence of an oligopeptide in the mucosal fluid, and the replacement of Na ions by K ions. The transport rate of TRH from mucosal side to serosal side was extremely high compared with that in the opposite direction, and the absorption from the upper small intestine was much superior to that from the lower part. The quantity of the TRH permeation from serosal to mucosal fluids in the experiments of non-everted sac could not be disregarded. That may be due to the existence of the route of a passive diffusion mechanism observed at least in the experiment of the *in vitro* everted sac.

Some investigators have reported the influence of molecular structure on the transport and hydrolysis of oligopeptides in the small intestine. Matthews described that if the amino-terminal or the carboxyl-terminal group of oligopeptides is

substituted, affinity to the carrier for transport is reduced, and also the presence of a γ-linkage in a molecule reduces affinity to the carrier for peptide transport, and also retards hydrolysis.[19] It would be the reason why the permeation from mucosal to serosal fluids was so slow compared with oligopeptides or β-lactam antibiotics. However, little or no absorption of TRH from middle or lower part of small intestine in *in situ* experiment suggests that the passive diffusion mechanism may not be the main route for the absorption of TRH. Otherwise, apparent TRH absorption could be found from middle and lower part of small intestine in *in situ* experiments.

From these results, it is concluded that TRH would be transported across the membrane of the small intestine mainly by carrier-mediated systems.

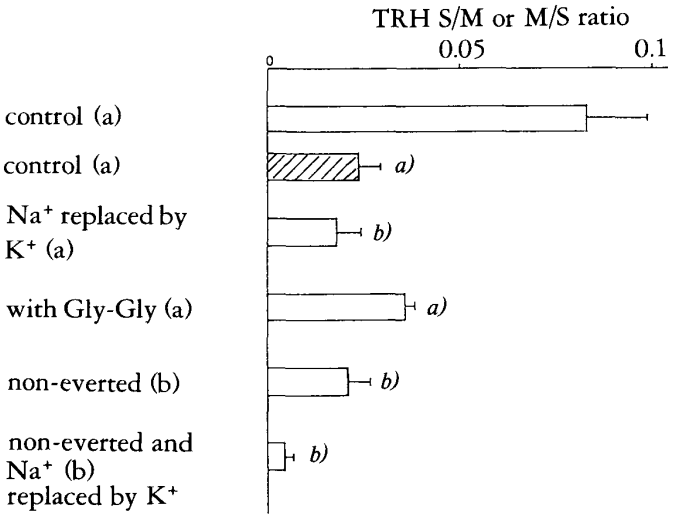

FIG. 5. *Transfer of TRH through Everted Sac of Rat Small Intestine*

Initial TRH-T concentration in mucosal side is 1 ng/ml and gly-gly is 10 ng/ml.

Data point represents the average of the three experiments: the horizontal bar represents the standard error of the mean.

(a) shows S/M ratio: final serosal-to-mucosal fluids concentration ratio.

(b) shows M/S ratio: final mucosal-to-serosal fluids concentration ratio. The significant difference to the control (upper small intestine) is expressed with a) ($p < 0.05$) and b) ($p < 0.01$).

upper small intestine, lower small intestine.

Acknowledgements The authors would like to acknowledge the continuing guidance of Drs. Y. Nagawa and T. Shimamoto, Takeda Chemical Industries Ltd.

REFERENCES

1) E. D. Haigler, J. M. Hershman and J. A. Pittman: Response to orally administered synthetic thyrotropin-releasing hormone in man, *J. Clin. Endocrinol. Metab.*, **35**, 631–635 (1972).
2) S. Yokohama, K. Yamashita, H. Toguchi and N. Kitamori: Radioimmunoassay of thyrotropin-releasing hormone (TRH) in rat, dog, and human blood, *J. Pharm. Dyn.*, **6**, 627–636 (1983).
3) S. Yokohama, K. Yamashita, H. Toguchi, J. Takeuchi and N. Kitamori: Absorption of thyrotropin-releasing hormone (TRH) after oral administration of TRH tartrate monohydrate in the rat, dog and human, *J. Pharm. Dyn.*, **7**, 101–111 (1984).
4) W. T. Agar, F. J. R. Hird and G. S. Sidhu: The active absorption of amino-acids by the intestine, *J. Physiol.* (London), **121**, 255–263 (1953).
5) D. S. Wiggans and J. M. Johnston: The absorption of peptides, *Biochim. Biophys. Acta*, **32**, 69–73 (1959)
6) I. L. Craft, D. Geddes, C. W. Hyde, I. J. Wise and D. M. Matthews: Absorption and malabsorption of glycine and glycine peptides in man, *Gut*, **9**, 425–437 (1968).
7) S. A. Adibi and D. W. Mercer: Protein digestion in human intestine as reflected in luminal, mucosal, and plasma aminoacid concentrations after meals, *J. Clin. Invest.*, **52**, 1586–1594 (1973).
8) J. M. Addison, D. Burston, J. A. Dalrymple, D. M. Matthews, J. W. Payne, M. H. Sleisenger and S. Wilkinson: A common mechanism for transport of di- and tripeptides by hamster jejunum *in vitro*, *Clin. Sci. Mol. Med.*, **49**, 313–322 (1975).
9) D. M. Matthews: Intestinal absorption of peptides, *Physiol. Rev.*, **55**, 537–608 (1975).
10) N. Nakajima, K. Yoshida and Y. Asahi: Physicochemical properties, stabilities and analysis of TRH L-tartrate (TRH-T), *J. Takeda Res. Lab.*, **35** (1/2), 23–33 (1976).
11) O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall: Protein measurement with the folin phenol reagent, *J. Biol. Chem.*, **193**, 265–275 (1951)
12) T. Noguchi, C. Takahashi, T. Kimura, S. Muranishi and H. Sezaki: Mechanisms of the intestinal absorption of durgs from oil-in-water emulsion. IV, Absorption of lipid-soluble dyes from tributyrin and triolein emulsions in rat small intestine, *Chem. Pharm. Bull.*, **23**,

775–781 (1975).
13) T. H. Wilson and G. Wiseman: The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface, *J. Physiol.* (London), **123,** 116–125 (1954).
14) H. Klostermeyer and R. E. Humbel: The chemistry and biochemistry of insulin, *Angew. Chem. Int. Ed. Engl.,* **5,** 807–822 (1966).
15) C. B. Coutinho, H. E. Spiegel, S. A. Kaplan, M. Yu, R. P. Christian, J. J. Carbon, J. Symington, J. A. Cheripko, M. Lewis, A. Tonchen and T. Crews: Kinetics of absorption and excretion levodopa in dogs, *J. Pharm. Sci.,* **60,** 1014–1018 (1971).
16) R. F. Crampton, M. T. Lis and D. M. Matthews: Sites of mucosal absorption and hydrolysis of two dipeptides by rat small intestine *in vitro, Clin. Sci.,* **44,** 583–594 (1973).
17) T. Kimura, H. Endo, M. Yoshikawa, S. Muranishi and H. Sezaki: Carrier-mediated transport systems for aminopenicillins in rat small intestine, *J. Pharm. Dyn.,* **1,** 262–267 (1978).
18) D. M. Matthews: Absorption of peptides by mammalian intestine, "Peptide Transport in Protein Nutrition," ed. by D. M. Matthews and J. W. Payne, North Holland American Elsevier, Amsterdam and New York, 1975, pp. 61–146.
19) D. M. Matthews and S. A. Adibi: Progress in gastroenterology, "Peptide absorption," *Gastroenterology,* **7,** 151–161 (1976).