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Enhancement of intestinal transport of thyrotropin-releasing hormone via a carrier-mediated transport system by chemical modification with lauric acid

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Abstract

The transport characteristics of thyrotropin-releasing hormone (TRH) and its chemically modified derivative with lauric acid (Lau-TRH) across the rat small or large intestine were estimated by means of an in vitro everted sac experiment. Both compounds were especially absorbed from the upper small intestine. The penetration of TRH across the upper small intestine was significantly increased by conjugation with lauric acid. Lau-TRH administered to the mucosal side appeared as a native TRH form in the serosal side. On the other hand, a temperature dependency and a directional difference in the transfer rates of these compounds were observed in the everted and non-everted sacs of the upper small intestine. Moreover, the penetration of TRH and Lau-TRH across the upper small intestine was inhibited by 0.25 mM 2,4-dinitrophenol and 10 mM glycylglycine. In addition, Lau-TRH was very stable in the cytosolic fraction of the small intestinal mucosa, while it was gradually converted to the native TRH in the brush-border membrane (BBM) fraction. The binding amounts of TRH to the BBM were remarkably enhanced by the lauric acid conjugation; however, its binding was nonspecific. Therefore, it was suggested that Lau-TRH rapidly bound to the BBM in the small intestine, where Lau-TRH is converted to TRH, and this released TRH is efficiently transported by an oligopeptide transporter which exists in the upper small intestine.

Keywords: Thyrotropin-releasing hormone; Lauric acid conjugated thyrotropin-releasing hormone; Intestinal absorption; Oligopeptide transporter; Carrier-mediated transport; Absorption enhancer

1. Introduction

Thyrotropin-releasing hormone (TRH: L-pyroglutamyl-L-histidyl-L-proline amide) is the hypothalamic peptide that regulates the synthesis and the secretion of thyrotropin from the anterior pituitary gland [1]. TRH has been shown to have not only a variety of endocrine activities, but also central nervous system-related biological activities. Recently, TRH has attached much attention as a potential drug for the management of various neurologic and neuropsychiatric disorders including depression, brain injury, acute spinal trauma, schizophrenia and Alzheimer's disease [2,3]. Although orally administered TRH has been reported to enhance thyroid stimulating hormone release in man, the absolute bioavailability is very low in man (1–2%), in rats (0.2–1.5%), and in dogs (4–13%) [4]. This low bioavailability may be attributed to its low lipophilicity.

Yokohama et al. reported that TRH was absorbed only from the upper region of the small intestine [5]. Based on the results of an in vitro everted sac experiment, TRH was suggested to be transported by a carrier-mediated mechanism. Recently, Nicklin et al. [6] and Walter and Kissel [7] found that TRH was transported across the Caco-2 monolayers by an active transport mechanism. In contrast, some investigators failed to find any direct evidence for the carrier-mediated transport of TRH in this system [8]. On the other hand, it was suggested that the other peptide mimetic compounds such as amino-β-lactam antibiotics [9–12,37] and angiotensin-converting enzyme inhibitors [13] were transported by a di/tripeptide carrier mechanism, as was the case of TRH.

In a previous report, in order to improve the intestinal absorption of TRH, we have synthesized a novel lipophilic derivative of TRH (Lau-TRH: Fig. 1) by chemical attach-

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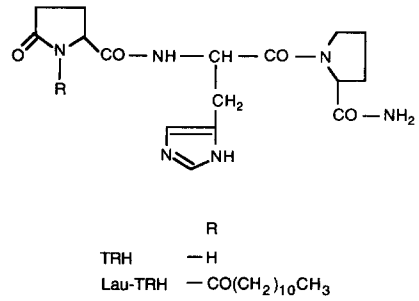

Fig. 1. Chemical structures of TRH and Lau-TRH.

ment of lauric acid to N-terminal pyroglutamyl group [14]. Lau-TRH retained 64 and 81% of the original central nervous system activity and thyrotropin-stimulating hormone releasing activity, respectively [14]. In rat in situ closed intestinal loop experiments, the intestinal absorption of TRH was significantly increased by a chemical attachment of lauric acid [15]. However, the absorption mechanism of Lau-TRH was unclear.

In the present study, we examined the transport characteristics of Lau-TRH across the intestine using an in vitro everted sac experiment in rats. In addition, we also investigated the regional differences of Lau-TRH on the intestinal absorption and its degradation characteristics in the brush-border membrane and cytosolic fractions of the rat small intestine.

2. Materials and methods

2.1. Chemicals

TRH was purchased from Peptide Institute, Inc. (Osaka, Japan). Lau-TRH was synthesized as reported previously [14]. Glycylglycine (Gly-Gly) and EDTA were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 2,4-Dinitrophenol (2,4-DNP) and sodium caprate was obtained from Wako Pure Chemicals, Ltd. (Osaka, Japan). *O*-n-Lauryl-β-D-maltopyranoside (LM) was from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the finest grade available without further purification.

2.2. Transport of TRH and Lau-TRH across the intestine

The transport characteristics of TRH and Lau-TRH across the intestinal mucosal membrane was examined using the everted sac in rats [16]. In brief, male Wistar rats (Japan SLC, Hamamatsu, Japan) (250–300 g) were fasted for about 18 h prior to the experiments but given water ad libitum. The rats were anesthesized with sodium pentobarbital (i.p., 32 mg/ml) and sacrificed by exsanguination from the inferior vena cava. The small intestine was quickly removed and separated by three segments, such as the upper, middle and lower regions at each 5-cm long. Each segment was rinsed with saline and everted, and then each end of the segment was ligated to silicon tubing with silk thread. As a serosal fluid, 0.5 ml of the isotonic phosphate buffer containing 10 mM D-glucose (pH 7.4) was introduced. The everted sac was placed in the isotonic phosphate buffer (pH 6.5) containing 10 mM D-glucose with 0.1 mM drug bubbled with 95% O_2 and 5% CO_2 at 37°C. The whole serosal solution was periodically collected and immediately replaced by the buffer solution.

The viability of gut sacs during the test period was monitored by measuring the transport of Trypan blue dye. There was minimal transport of dye during the incubation.

For the determination of TRH or Lau-TRH, 100 μl of the sample solutions were added to 50 μl of acetonitrile to terminate the reaction. The resulting mixture was centrifuged at 12000 rev/min for 5 min. Thirty microlitres of the supernatant were injected into HPLC.

The apparent permeability coefficient (P_{app}) of each drug was calculated according to the following equation:

$$P_{app} = \frac{dC}{dt} \cdot \frac{V}{C_0 \cdot A}$$

where, dC/dt is the change in concentration per unit time (μmol/ml/s), V is the volume of the serosal fluid (ml), C_0 is the initial concentration of the mucosal drug solution (μmol/ml) and A is the apparent surface area of the intestinal membrane (cm^2). The P_{app} values were calculated using data points up to which sink condition seemed to be maintained.

2.3. Preparation of brush-border membrane

Brush-border membranes were isolated from the upper and middle small intestine of male Wistar rats (250–300 g) according to the $CaCl_2$ precipitation method [17]. All steps were performed on ice or at 4°C. The purity of the membrane was evaluated according to the enrichment of alkaline phosphatase, a marker enzyme for brush-border membrane. The specific activity of this enzyme was increased 10-fold in the final membrane suspension as compared with the crude homogenate of the intestinal scrapping. The brush-border membrane was diluted to a protein concentration of 2 mg/ml. The protein concentration in the brush-border membrane was determined by the method of Lowry et al. using bovine serum albumin fraction V as a standard [18].

2.4. Preparation of the cytosolic enzyme solution

The cytosolic fraction of the intestinal mucosal homogenate was prepared according to the method of Ganapathy et al. with a slight modification [19]. The activity of lactate dehydrogenase, a maker enzyme of the cytosol, was measured by the method of Harauchi and Yoshizaki [20]. The activity in the final preparation was about 3-fold higher than that in the original homogenate. Moreover, the activity of alkaline phosphatase, a maker enzyme of

brush-border membrane, decreased and was about 80 times as low as that in the crude homogenate. The cytosolic fraction was diluted with the experimental buffer solution to give a protein concentration of 2 mg/ml. Hence, the contamination of brush-border membrane was minimal.

2.5. Preparation of the intestinal luminal fluid

The small intestinal fluid was collected from the rat according to the method of our previous report [21]. Three male Wistar rats, weighing 250–300 g, were anesthetized with an i.p. injection of sodium pentobarbital (45 mg/kg). After the injection of 10 ml of distilled water into the small intestine, cannulated with silicon tubing (3 mm i.d. × 5 mm o.d.) at the proximal duodenum and the ileocecal junction, rats were left for 5 min and the content was washed out by infusing 5 ml of 0.15 M NaCl and the eluate was collected. This eluate was extracted five times with two-fold volumes of methylene chloride to remove lipids that may interfere with the analysis of TRH and Lau-TRH by HPLC. This extract was diluted with 0.15 M NaCl to yield a protein concentration of 2 mg/ml and used the small intestinal fluid.

2.6. Degradation of TRH and Lau-TRH in the brush-border membrane and the cytosol fractions

The stability of TRH and Lau-TRH in the brush-border membrane and the cytosol fractions was examined by incubating 200 μl of a given concentration of the brush-border membrane (2 mg protein/ml) or the cytosol (2 mg/ml), which was preincubated at 37°C for 15 min, with 200 μl of 0.2 mM TRH or Lau-TRH. At an appropriate time, 50 μl was withdrawn from the incubation mixture, to which was added 100 μl of acetonitrile, to remove tissue proteins, thereby terminating the reaction. The resulting mixture was centrifuged for 10 min to remove precipitated protein. Thirty microlitres of the supernatant were injected into the HPLC.

2.7. Binding of TRH and Lau-TRH binding to the brush-border membrane

Binding properties of TRH and Lau-TRH to the brush-border membrane were evaluated according to the method of Saitoh et al. with a slight modification [36]. One hundred microlitres of drug solution (0.2 mM) was added to 100 μl of membrane suspension (2 mg protein/ml) at 4°C for 30 min. The resulting mixture was centrifuged at 1200 rev/min at 4°C for 20 min and 50 μl of the supernatant fluid was injected into the HPLC.

2.8. HPLC conditions

TRH and Lau-TRH were assayed by a gradient HPLC system (Hitachi L-6200, Hitachi, Tokyo, Japan) and a data processor (Hitachi AS-400 Intelligent Autosampler) using a C_{18}-column (4.6 × 150 mm) of YMC-pack Protein-RP. The mobile phase was a mixture of 0.1% trifluoroacetic acid (mobile phase A) and acetonitrile (mobile phase B). The gradient system was programmed by linearly increasing the proportion of mobile phase B from 0 to 70% within 30 min. The gradient mobile phase was run at a flow rate 0.8 ml/min. The UV detector was set at 210 nm.

2.9. Statistical analysis

Results are expressed as the mean ± S.E. Statistical data analyses were assessed using the Student's *t*-test with $P < 0.05$ as the minimal level of significance.

3. Results

3.1. Transport of TRH and Lau-TRH across the intestine

The penetrated amounts of TRH through the upper small intestine were significantly increased by the introduction of lauric acid into TRH molecule (Fig. 2). Al-

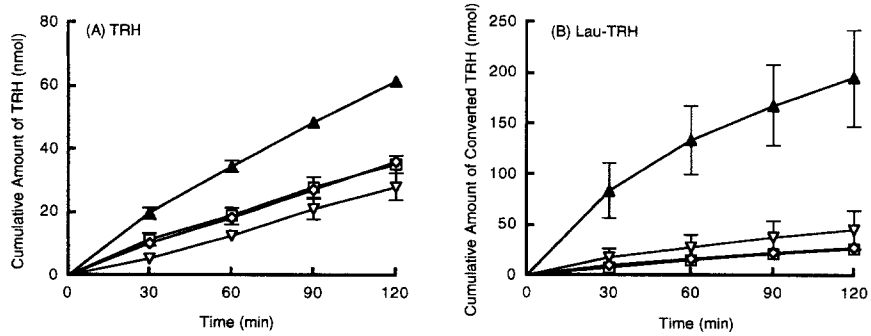

Fig. 2. Regional differences in the intestinal transport of (a) TRH and (b) Lau-TRH across the rat everted intestine. The transport characteristics of TRH and Lau-TRH across the intestinal mucosal membrane were examined using the everted sac in rats [16]. As a serosal fluid, 0.5 ml of the isotonic phosphate buffer containing 10 mM D-glucose (pH 7.4) was introduced. The everted sac (5 cm long) was placed in the isotonic phosphate buffer (pH 6.5) containing 10 mM D-glucose with 0.1 mM drug bubbled with 95% O_2 and 5% CO_2 at 37°C. The whole serosal solution was periodically collected and immediately replaced by the buffer solution. These samples were assayed by the HPLC. Results are expressed as the mean ± S.E. of three experiments. Keys: upper small intestine (▲); middle small intestine (◇); lower small intestine (▽); large intestine (□).

Table 1
Regional differences in apparent permeability coefficients of TRH and Lau-TRH across the various intestinal membrane of rat everted sacs

	P_{app} ($\times 10^{-6}$ cm/s) TRH	P vs. upper region	P_{app} ($\times 10^{-6}$ cm/s) Lau-TRH	P vs. upper region	P vs. TRH
Small intestine					
Upper region	9.22 ± 0.59		24.4 ± 4.40		< 0.05
Middle region	6.42 ± 0.30	< 0.02	4.50 ± 0.22	< 0.02	< 0.05
Lower region	5.07 ± 0.79	< 0.05	6.13 ± 2.25	< 0.02	n.s.
Large intestine	4.94 ± 0.22	< 0.01	3.84 ± 0.13	< 0.01	< 0.05

The everted sac was placed in the isotonic phosphate buffer (pH 6.5) containing 10 mM D-glucose with 0.1 mM TRH or Lau-TRH bubbled with 95% O_2 and 5% CO_2 at 37°C. The whole serosal solution was periodically collected and immediately replaced by the buffer solution. For the determination of TRH or Lau-TRH, 100 μl of the sample solutions were added to 50 μl of acetonitrile to terminate the reaction. These samples were analyzed by the HPLC. The apparent permeability coefficient (P_{app}) of each drug was calculated according to the following equation:

$$P_{app} = \frac{dt}{dC} \cdot \frac{V}{C_0 \cdot A}$$

where, dC/dt is the change in concentration per unit time (μmol/ml/s), V is the volume of the serosal fluid (ml), C_0 is the initial concentration of the mucosal drug solution (μmol/ml) and A is the apparent surface area of the intestinal membrane (cm^2). The P_{app} value was calculated using data points up to which sink condition seemed to be maintained. Results are expressed as the mean ± S.E. of three experiments. Statistical differences were calculated by Student's t-test.

Table 2
Effects of metabolic inhibitor, dipeptide, low temperature and polarity on the upper small intestinal transport of TRH and Lau-TRH in the rat everted sacs

Condition	P_{app} ($\times 10^{-6}$ cm/s) TRH	P vs. control	P_{app} ($\times 10^{-6}$ cm/s) Lau-TRH	P vs. control
Control	9.22 ± 0.59		24.4 ± 4.40	
Non-everted	4.66 ± 0.05	< 0.01	1.36 ± 0.17	< 0.01
25°C	6.66 ± 0.66	< 0.02	4.95 ± 0.94	< 0.05
10 mM Gly-Gly	6.65 ± 0.34	< 0.01	2.33 ± 0.16	< 0.02
0.25 mM 2,4-DNP	3.89 ± 0.14	< 0.01	2.30 ± 0.17	< 0.001

The everted sac was placed in the isotonic phosphate buffer (pH 6.5) containing 10 mM D-glucose with 0.1 mM TRH or Lau-TRH bubbled with 95% O_2 and 5% CO_2 under various conditions. The whole serosal solution was periodically collected and immediately replaced by the buffer solution. For the determination of TRH or Lau-TRH, 100 μl of the sample solutions were added to 50 μl of acetonitrile to terminate the reaction. These samples were analyzed by the HPLC. The apparent permeability coefficient (P_{app}) under various conditions was calculated by a similar equation to Table 1. Results are expressed as the mean ± S.E. of three experiments. Statistical differences were calculated by Student's t-test.

though Lau-TRH was very stable in the experimental solution, Lau-TRH was enzymatically converted to the native TRH in the intestinal mucosal surface and it appeared as a native TRH form in the serosal side (data not shown). Fig. 2 shows the regional differences in the intestinal transport of TRH and Lau-TRH across the rat everted intestine. Table 1 shows the apparent permeability coefficients of TRH and Lau-TRH across various intestinal regions in rat everted sacs. The absorption of both compounds from the upper small intestinal region were significantly higher than that of other regions. However, no significant difference was observed for TRH or Lau-TRH transport across the middle and lower small intestine, and the large intestine.

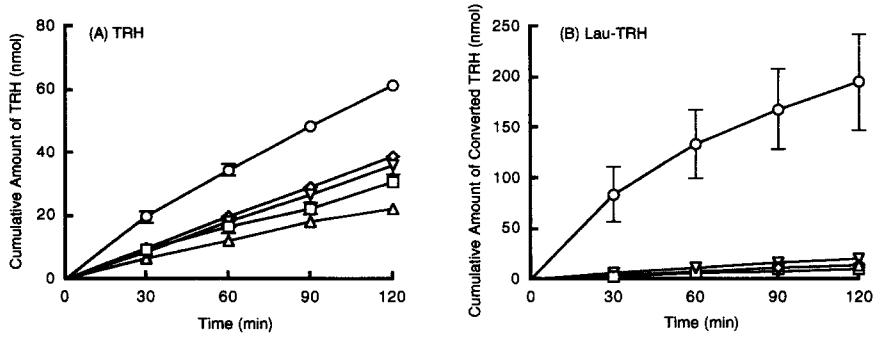

Fig. 3. Effect of various conditions on the transport of TRH (a) and Lau-TRH (b) across the rat upper small intestine. Experimental system was similar to Fig. 2. In certain experiments, 10 mM glycylglycine (Gly-Gly) or 0.25 mM 2,4-dinitrophenol (2,4-DNP) were added to the mucosal fluid. Results are expressed as the mean ± S.E. of three experiments. Keys: control (○); non-everted intestine (□); 25°C (▽); 10 mM Gly-Gly (◇); 0.25 mM 2,4-DNP (△).

3.2. Contribution of carrier-mediated mechanisms to the small intestinal absorption of TRH and Lau-TRH

In an attempt to clarify the transport mechanisms of TRH and Lau-TRH across the upper small intestine, we examined the effects of metabolic inhibitor, dipeptide, low temperature and polarity (directional difference) on their upper small intestinal absorption. Fig. 3 shows the transport profiles of TRH and Lau-TRH across the upper small intestine under various conditions. The mucosal-to-serosal flux of both compounds was significantly faster than the serosal-to-mucosal flux in the upper small intestine. Similar results were observed in the presence of dipeptide (Gly-Gly) and metabolic inhibitor (2,4-DNP), and under the low temperature condition (Table 2). In particular, the inhibitory effects of Lau-TRH transport under these conditions were more predominant than those of TRH. These results suggest that Lau-TRH is also transported across the upper small intestine via an oligopeptide transporter by a carrier-mediated process as well as TRH.

3.3. Effect of lipophilic modification and/or the use of absorption enhancers on improving TRH transport across the intestinal membrane

We compared the permeation enhancing efficacy by lipophilic modification of TRH with those by various absorption enhancers (Fig. 4). In the upper small intestinal region, all the absorption enhancers tested in this experiment failed to enhance transport of TRH across the intestinal membrane, while, in the large intestine, a marked increase in its P_{app} values was observed by the addition of these absorption enhancers. On the other hand, lipophilic modification enhanced the transport of TRH across the upper small intestine. However, in the middle small intestine and large intestine, no enhancing effect was observed in the permeation of TRH by lipophilic modification with lauric acid.

3.4. Stability experiments

In order to examine the degradation properties of Lau-TRH on the brush-border membrane in the upper small intestine, we investigated the stability of Lau-TRH in the brush-border membrane fraction. Thirty percent of Lau-TRH was gradually converted to TRH up to 60 min (Fig. 5). The total recovery percentage of Lau-TRH and TRH was approx. 100%, and no degradation products were observed except for TRH. A similar result was obtained in the middle small intestinal region (data not shown). On the other hand, Lau-TRH was extremely stable in the cytosolic fraction and the intestinal fluid (data not shown). These results indicated that Lau-TRH was converted to TRH by the brush-border membrane enzymes of the small intestinal mucosa and that there is no regional difference in the conversion of Lau-TRH to TRH in the rat small intestine.

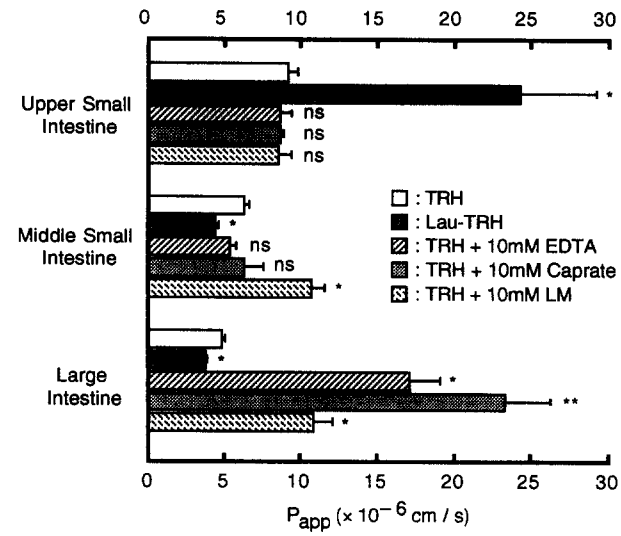

Fig. 4. Effects of lipophilic modification and/or the use of absorption enhancers on improving TRH transport across the intestinal membrane. The apparent permeability coefficient (P_{app}) of each drug was calculated according to the following equation: $P_{app} = \frac{dC}{dt} \cdot \frac{V}{C_0 \cdot A}$ where, dC/dt is the change in concentration per unit time (μmol/ml/s), V is the volume of the serosal fluid (ml), C_0 is the initial concentration of the mucosal drug solution (μmol/ml) and A is the apparent surface area of the intestinal membrane (cm^2). Results are expressed as the mean ± S.E. of three experiments. Statistical differences were calculated by Student's t-test. LM: O-n-lauryl-β-D-maltopyranoside.

3.5. Binding characteristics of Lau-TRH to the BBM

Fig. 6 shows the binding properties of TRH and Lau-TRH to the BBM of the upper small intestine. The binding amount of Lau-TRH to the BBM was significantly higher than that of TRH. In addition, no significant difference in the binding of Lau-TRH to the BBM was observed be-

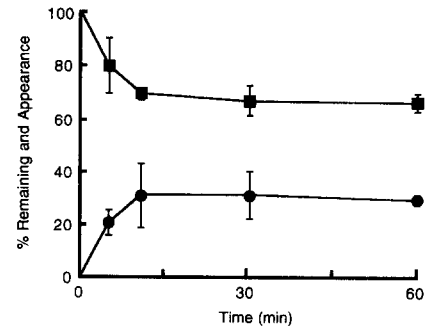

Fig. 5. Degradation profile of Lau-TRH and corresponding appearance of TRH in the brush-border membrane prepared from the upper small intestine in rats. The stability of Lau-TRH in the brush-border membrane was examined by incubating 200 μl of a given concentration of the brush-border membrane (2 mg protein/ml), which was preincubated at 37°C for 15 min, with 200 μl of 0.2 mM Lau-TRH. At an appropriate time, 50 μl was withdrawn from the incubation mixture, to which was added 100 μl of acetonitrile, to remove tissue proteins, thereby terminating the reaction. The resulting mixture was centrifuged for 10 min to remove precipitated protein. These samples were assayed by the HPLC. Keys: TRH (●); Lau-TRH (■).

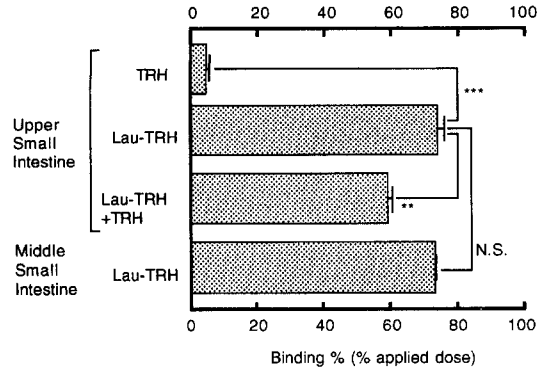

Fig. 6. Binding of TRH and Lau-TRH to the small intestinal brush border membrane. Brush-border membranes were isolated from the upper and middle small intestine of male Wistar rats (250–300 g) according to the $CaCl_2$ precipitation method [17]. Binding properties of TRH and Lau-TRH to the brush-border membrane were evaluated according to the method of Saitoh et al. with a slight modification [36]. One hundred microliters of drug solution (0.2 mM) was added to 100 μl of membrane suspension (2 mg protein/ml) at 4°C for 30 min. Results are expressed as the mean ± S.E. of three experiments. Statistical differences were calculated by Student's *t*-test.

tween the upper and middle small intestinal region. The binding amount of Lau-TRH to the upper small intestinal BBM was reduced by the addition of excess amount of TRH. Thus, it was suggested that Lau-TRH competed with TRH in binding to the upper small intestinal BBM.

4. Discussion

In our previous studies, we have synthesized lipophilic derivatives of various peptides as such as tetragastrin [22,23], calcitonin [24] and insulin [25,26] by chemical modification with fatty acids. In preceding papers, we have reported that these acyl-peptides were more permeable across the intestine than the unmodified peptides while retaining their biological activities [23,25,26]. However, these peptides were absorbed from the intestine by the passive transport system because they consist of more than 4 amino acid residues. Therefore, it was demonstrated that the intestinal absorption of these peptides was improved due to the increase in their lipophilicity.

In this study, we found that the transport of Lau-TRH across the upper small intestine was much higher than that of TRH. This result was partly due to its lipophilicity compared with TRH as assessed by HPLC and partition coefficient experiment. We previously reported that the partition coefficients of Lau-TRH were 1.91 and 2.14 in n-octanol/phosphate buffer and $CHCl_3$/phosphate buffer, respectively, whereas those of TRH were 0.068 and 0.088, respectively [14]. In HPLC analysis, the retention time of Lau-TRH (21.3 min) was delayed compared with that of TRH (8.01 min). One possible mechanism of enhancing Lau-TRH transport is by conversion to lauric acid, a degradation product by Lau-TRH, which can enhance the permeability of TRH in the gut. However, this mechanism was unlikely, as even if all lauric acid was released from Lau-TRH, the concentration of this fatty acid would be too low to display its absorption enhancing properties in the gut.

The transport characteristics of di/tripeptides into the intestinal epithelial cells is an active process which is mediated by an oligopeptide transporter localized in the BBMs. The transport efficacy of TRH across the upper small intestine was relatively low as compared with other di/tripeptides and some amino-β-lactam antibiotics. Matthews suggested that the lack of C-terminal carboxyl groups of di/tripeptide and the presence of an intramolecular γ-linkage reduced its affinity to the oligopeptide transporter [27]. Bai et al. reported the structural requirement of dipeptide to interact with the oligopeptide transporter using several dipeptide analogs, suggested that the hydrophobic interaction between dipeptides and the transporter was important to improve their transport efficiency [28,29]. Therefore, the introduction of lauric acid into the N-terminal pyroglutamyl group of TRH might improve its low affinity to the oligopeptide transporter and enhanced TRH transport across the upper small intestine.

In a previous report, we studied the absorption characteristics of [^{125}I]Lau-TRH using an in situ rat closed intestinal loop method [15]. The absorption of [^{125}I]Lau-TRH from the small intestine was decreased in the presence of 100-fold excess TRH, suggesting that Lau-TRH was partly absorbed by a carrier-mediated transport system via an oligopeptide transporter. In order to study the further transport mechanism of Lau-TRH across the intestinal membrane, in the present study, we investigated the transport characteristics of TRH and Lau-TRH using an in vitro everted intestinal sac experiment. In this experimental system, Lau-TRH was specifically transported as a form of native TRH across the upper small intestine (Fig. 2). In addition, its transport was markedly inhibited under various conditions, such as the addition of 0.25 mM 2,4-DNP and 10 mM of Gly-Gly, and the low temperature (Fig. 3). Therefore, these findings suggest that chemical modification of TRH with lauric acid enhanced its transport across the upper small intestine by a carrier-mediated transport system, which are in good agreement with our previous report [15].

In a preliminary study, we synthesized chemically modified phenylalanyl-glycine (Phe-Gly) derivatives with various fatty acids, and their uptake characteristics into the brush-border membrane vesicles (BBMVs). In the presence of an inwardly H^+ gradient, the initial uptake of Phe-Gly by the BBMVs was enhanced by the covalently attachment of fatty acids to N-terminal of Phe-Gly. Moreover, their uptake was stimulated by the countertransport effects of other dipeptides, and was inhibited by other dipeptides (unpublished data). These detailed results will be described in a future report.

The negative effect of absorption enhancers (10 mM

conc.) was observed in the upper small intestinal transport of TRH (Fig. 4). These results were corresponded to a previous report that the intestinal absorption of 5-fluorouracil, which was absorbed from the small intestine by a carrier-mediated mechanism, failed to be enhanced by absorption enhancer [30]. On the contrary, the large intestinal transport of TRH, whose mechanism was a passive diffusion, was significantly improved by the absorption enhancers such as EDTA, caprate and *O*-n-lauryl-β-D-maltopyranoside (LM) (Fig. 4). LM, an alkylsaccharide, has recently been found to have absorption enhancing activity and to exhibit the absorption enhancing effect even at considerably low concentration with low local irritation in the gastrointestinal tract [31]. In general, in the case of drugs transported by a passive diffusion mechanism, the absorption enhancing actions by various absorption enhancers, such as caprate and LM, in the large intestine were more predominant than those in the small intestine [31]. Therefore, this finding was in good agreement with the previous reports [30].

We found only TRH but not Lau-TRH in the serosal fluid after administration of Lau-TRH into the mucosal fluid. Furthermore, Lau-TRH was extremely stable in the small intestinal fluid and cytosol, indicating a little susceptibility of Lau-TRH to serine proteases, such as chymotrypsin and trypsin, and prolylendopeptidase. On the other hand, Lau-TRH was gradually converted to TRH in the intestinal BBM fraction (Fig. 5). Accordingly, unidentified enzymes in the BBM may be responsible for the degradation of Lau-TRH.

We demonstrated that the binding affinity of Lau-TRH to BBM was significantly higher than that of TRH. In addition, no regional difference on its BBM binding was observed between the upper and middle small intestinal BBM because its binding was nonspecific due to the increased lipophilicity of TRH by lipophilic modification (Fig. 6). However, the percentage of Lau-TRH binding to the upper small intestinal BBM was decreased by the addition of excess TRH (Fig. 6). Thus, it was suggested that Lau-TRH was partly bound to the oligopeptide transporter in the upper small intestine. Although the binding mechanism of Lau-TRH is unclear, this high binding to the intestinal BBM might play a part role on the enhanced transport across the upper small intestine.

Recently, a cDNA encoding an oligopeptide transporter (PepT1) was isolated from the rabbit [32], rat [33] and human [34,35] small intestine. Thus, the use of this expression system would give further detailed findings for the carrier-mediated transport of TRH and its analogs and/or prodrugs by the oligopeptide transporter.

In conclusion, the above results suggested that Lau-TRH is highly transported from the upper small intestine by a carrier-mediated transport system via an oligopeptide transporter and the high affinity of the lipophilic peptide to lipid components of BBM might be one of the critical factors for the first step of the carrier-mediated transport. Furthermore, the acylation might improve the transport of various type of peptides and proteins which are transported by not only a passive diffusion but also a carrier-mediated mechanism.

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