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Improvement of Intestinal Absorption of Thyrotropin-releasing Hormone by Chemical Modification with Lauric Acid

KEIGO YAMADA, MASAHIRO MURAKAMI, AKIRA YAMAMOTO, KANJI TAKADA AND SHOZO MURANISHI

Department of Biopharmaceutics, Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607, Japan

Abstract—Intestinal absorption of ^{125}I-labelled lauryl thyrotropin-releasing hormone (Lau-TRH), a novel lipophilic derivative of TRH, was examined by rat in-situ closed intestinal loops. At a dose of 1 μmol per rat into the small intestine, a significant increase in percent of dose in plasma radioactivity of Lau-TRH was observed in comparison with that of TRH. A dose-dependent decrease in percent of dose in plasma radioactivity of TRH was noted, suggesting a saturable process of TRH transport. In contrast, the percent of dose in plasma radioactivity of Lau-TRH increased with increasing dose of Lau-TRH. The stability of TRH and Lau-TRH was studied in plasma and rat small intestinal homogenates. Lau-TRH was more stable than TRH in rat plasma. These results suggest that chemical modification of TRH with lauric acid may not only increase the lipophilicity of TRH but also reduce the degradation of TRH, resulting in the increased plasma radioactivity of TRH. On the other hand, Lau-TRH was gradually converted to TRH in the intestinal mucosal homogenate. These findings indicate that chemical modification of TRH with lauric acid might be a useful approach for improving the intestinal absorption of this peptide.

The availability of proteins and peptides with therapeutic potential has increased dramatically in the past decade as a consequence of recombinant DNA technology and the emergence of the biopharmaceutical industry (Lee 1986). Currently, polypeptide drugs are administered by intravenous, intramuscular or subcutaneous routes. From a practical point of view, oral administration is the route of choice. However, oral delivery of peptides and proteins is not always possible, as low gastric pH, proteolytic enzymes in the intestine and poor mucosal permeability to large molecules are all potential barriers to their absorption (Banga & Chien 1988; Davis 1989; Lee & Yamamoto 1990). Consequently, peptides and proteins have been co-administered with absorption-enhancing agents or enzyme inhibitors in order to promote passage through gastrointestinal epithelial barriers and to reduce degradation in the gut (Lee & Yamamoto 1990). These absorption promoters and protease inhibitors can enhance the absorption of normally non-absorbed molecules from the gastrointestinal tract (Lee & Yamamoto 1990). However, it is inevitable that these substances may cause membrane damage and local irritation.

A potentially useful approach to solving these delivery problems may be chemical modification of peptides and proteins to produce prodrugs and analogues. Thus, this approach may protect peptides against degradation by peptidases and other enzymes present at the mucosal barrier and render the peptides and proteins more lipophilic, resulting in increased bioavailability.

Thyrotropin-releasing hormone (TRH: L-pyroglutamyl-L-histidyl-L-prolinamide), is the hypothalamic peptide that regulates the synthesis and secretion of thyrotropin from the anterior pituitary gland. Since its discovery in 1969, TRH has been shown to have not only a variety of endocrine and central nervous system-related biological activities, but also potential as a drug in the management of various neurologic and neuropsychiatric disorders including depression, brain injury, acute spinal trauma and schizophrenia (Griffiths 1985). Although orally administered TRH has been reported to enhance thyroid stimulating hormone release in man, the absolute bioavailability of TRH is very low in man (1–2%), in rats (0·2–1·5%) and in dogs (4–13%) (Yokohama et al 1984a). This low bioavailability may be attributed to the low lipophilicity of TRH.

Recently, Bundgaard & Møss (1990) synthesized various new prodrugs of TRH to improve its bioavailability. They reported that *N*-octyloxycarbonyl derivatives of TRH showed a high penetrating capacity in human skin samples (Møss & Bundgaard 1990a) whereas *N*-alkoxycarbonyl derivatives of TRH did not improve the penetration of TRH across the jejunal, ileal and colonic segments of the rat (Møss et al 1990). In our previous report, we described the synthesis of a novel lipophilic derivative of TRH (Lau-TRH) by chemical attachment of lauric acid to the *N*-terminal pyroglutamyl group of TRH to improve the oral bioavailability of TRH (Muranishi et al 1991). It was demonstrated that this new derivative of TRH possesses higher lipophilicity relative to TRH, as assessed by HPLC and partition coefficient experiments. Since we have also synthesized new lipophilic insulin derivatives (palmitoyl insulins) (Hashimoto et al 1989), which were more permeable to the large intestinal membrane than the native insulin (Hashizume et al 1992), it is possible that the permeability of this lipophilic TRH derivative across various biomembranes is greater than that of native TRH.

In the present study, we determine whether this lipophilic derivative of TRH could be utilized to improve the bioavailability of TRH in the small intestine. We also describe the stability of Lau-TRH in comparison with native TRH in plasma and small intestinal mucosa.

Correspondence: S. Muranishi, Department of Biopharmaceutics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan.

Materials and Methods

Materials

Thyrotropin-releasing hormone (TRH) was purchased from Peptide Institute, Inc. (Osaka, Japan). Lau-TRH was synthesized as reported previously (Muranishi et al 1991). ^{125}I was purchased from New England Nuclear (Boston, MA, USA). Trifluoroacetic acid (TFA) and acetonitrile were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of reagent grade.

Preparation of test solution

The procedure of iodination of TRH or Lau-TRH was based on the chloramine T method (Hunter & Greenwood 1962; Greenwood et al 1963). The test solution (1 mL) containing [^{125}I]TRH or [^{125}I]Lau-TRH (0·1 μCi; [^{125}I]TRH, $3{\cdot}0\times10^{-6}$ μmol; [^{125}I]Lau-TRH, $3{\cdot}2\times10^{-5}$ μmol) was prepared in pH 6·5 phosphate-buffered saline (PBS).

Absorption experiments

Absorption experiments were performed by an in-situ closed loop method (Hashida et al 1984). Male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan), 280–300 g, with free access to water were fasted for 18 h before the absorption experiments and then were anaesthetized with sodium pentobarbitone (32 mg kg^{-1}, i.p.). The small intestine was exposed through a mid-line abdominal incision, and an intestinal loop (8 cm) was prepared by cannulation with silicon tubing (i.d., 3 mm: o.d., 5 mm) at the proximal and the distal ends of the upper small intestine. The bile duct was cannulated with polyethylene tubing (i.d., 0·5 mm: o.d., 0·8 mm) and bile was removed from the body during the experiment. The drug solution (1 mL) warmed at 37°C was introduced into the intestinal loop, which was closed by clipping with forceps at the cannulated position of each tubing. Blood samples were collected periodically through a polyethylene catheter (i.d., 0·5 mm: o.d., 0·8 mm) placed into a femoral artery. One hundred μL of plasma was rapidly separated and the plasma radioactivity was determined by a gamma counter (Aloka Auto Well Gamma System, ARC-500).

Stability of TRH and Lau-TRH in plasma

The stability of TRH and Lau-TRH in plasma was studied by incubating 9·9 mL of rat undiluted plasma, which had been preincubated at 37°C, with 100 μL of a TRH or Lau-TRH solution (initial concn 0·1 mM). The TRH and Lau-TRH solutions were prepared by dissolving these compounds in pH 6·5 PBS. At predetermined times up until 60 min, 1 mL was withdrawn from the incubation mixture, 10 mL of methanol was added and the mixture was centrifuged at 20000 *g* for 10 min. The supernatants were evaporated to dryness, and the residues were dissolved in 0·02 M HCl (200 μL). TRH and Lau-TRH were determined by an HPLC system using a Hitachi L-6200 intelligent pump, a Hitachi L-6000 pump, a Hitachi L-4000 UV detector operated at 230 nm, and a Hitachi AS-4000 Intelligent Autosampler. A reversed-phase YMC-AM 302 (ODS) column (4·6 × 150 mm) was used in the experiments, and the column was eluted with a linear gradient of acetonitrile (0–100%, 30 min) in 0·1% trifluoroacetic acid (TFA) at a flow rate of 1·0 mL min^{-1}. Under these conditions, TRH and Lau-TRH were eluted at 6·5 and 20·1 min, respectively.

Stability of TRH and Lau-TRH in gastrointestinal mucosa

The degradation of TRH and Lau-TRH in the mucosal homogenate of the small intestine was examined as follows. The upper small intestine was rinsed three times with 0·9% NaCl (saline). The mucosal tissue was scraped off with a glass slide, suspended in saline at 0°C, and homogenized using a Polytron homogenizer (Kinematica, GmbH, Switzerland) to a final concentration of 20% (w/v) homogenate. Three mL of TRH or Lau-TRH (1·0 mM) in pH 6·5 PBS was incubated with an equal volume of the homogenates at 37°C. At predetermined times up to 60 min, 1 mL was withdrawn from the incubation mixture, and TRH and Lau-TRH were determined as described above.

Results

Intestinal absorption of TRH and Lau-TRH

Fig. 1 shows the time course of plasma radioactivity following administration into the small intestine of TRH and Lau-TRH (1·0 μmol/rat). A significant increase in plasma radioactivity after [^{125}I]Lau-TRH dosing was observed in comparison with that following [^{125}I]TRH.

Fig. 2 shows percent of dose in plasma radioactivity after administration of [^{125}I]TRH into the small intestine at various doses. The percent of dose in plasma radioactivity decreased with increasing dose of [^{125}I]TRH, suggesting that the process of TRH absorption from the small intestine is saturable. In contrast, percent of dose in plasma radioactivity seemed to increase with increasing dose of [^{125}I]Lau-TRH (Fig. 3).

The relationship between dose of TRH or Lau-TRH and area under the plasma radioactivity vs time curve (AUC) after the intestinal absorption of TRH or Lau-TRH is shown in Fig. 4. The AUC of TRH decreased with increasing dose, whereas the reverse tendency was noted in the case of Lau-TRH.

Stability of TRH and Lau-TRH in rat plasma

Fig. 5 indicates the time course of degradation of TRH (a)

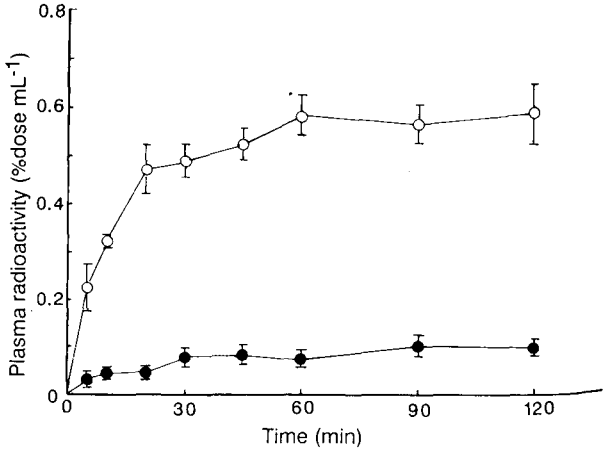

FIG. 1. Plasma radioactivity of TRH (●) and Lau-TRH (○) following administration into the small intestine. Dose: 1·0 μmol/rat. Symbols represent the mean ± s.e. (n = 4–5).

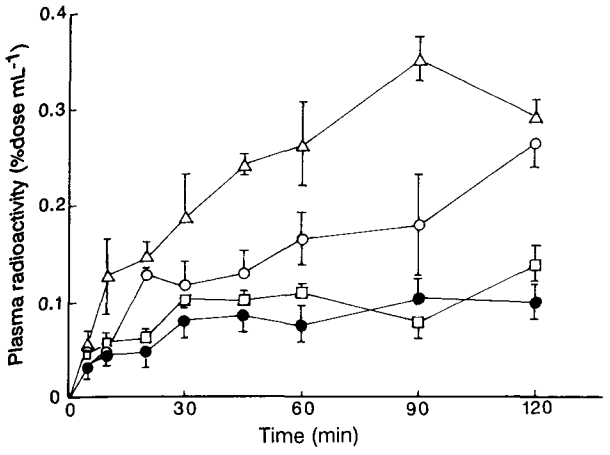

FIG. 2. Effect of dose on plasma radioactivity of TRH following administration into the small intestine. Dose: $3{\cdot}0 \times 10^{-6}$ (△), 0·25 (○), 1·0 (●), 10 μmol/rat (□). Symbols represent the mean ± s.e. (n = 4–5).

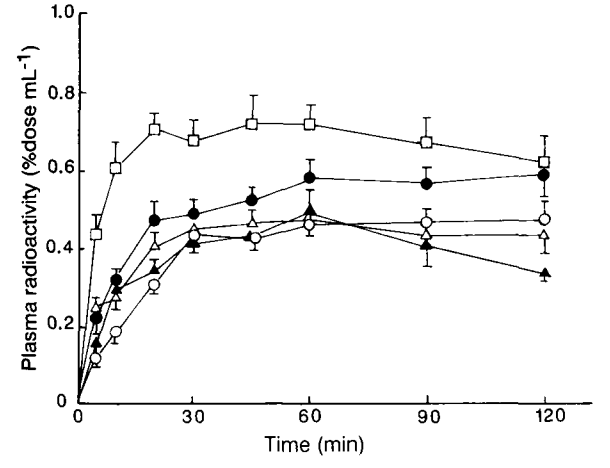

FIG. 3. Effect of dose on plasma radioactivity of Lau-TRH following administration into the small intestine. Dose: $3{\cdot}2 \times 10^{-5}$ (△), 0·1 (○), 0·5 (▲), 1·0 (●), 10 μmol/rat (□). Symbols represent the mean ± s.e. (n = 4–5).

and Lau-TRH (b) in rat plasma. The degradation of TRH was very rapid with less than 3% remaining at 60 min, whereas Lau-TRH was more stable with more than 80% remaining in rat plasma at 60 min. The half-lives for TRH and Lau-TRH proteolysis were 11·7 and 289·7 min (data not shown), respectively. However, TRH, a degradation product of Lau-TRH, was not detected during the incubation of Lau-TRH in plasma.

Stability of TRH and Lau-TRH in rat intestinal mucosal homogenate

The degradation of TRH and Lau-TRH in rat intestinal mucosal homogenate is shown in Fig. 6. TRH was very stable in 20% homogenate of the rat small intestinal mucosa with more than 80% of TRH still intact at 60 min. On the other hand, Lau-TRH was gradually degraded and converted to TRH during the incubation period. The total recovery percentage (remaining percent of Lau-TRH + appearance

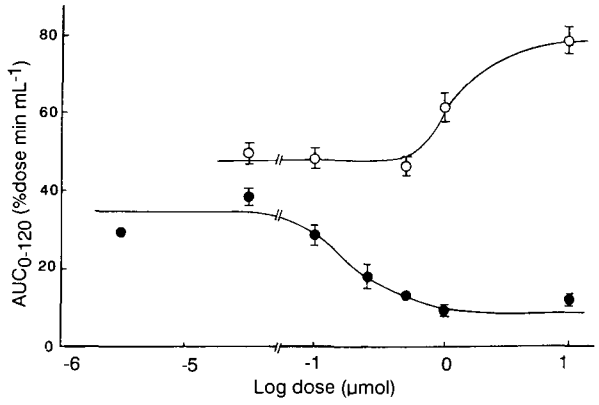

FIG. 4. Effect of dose on $AUC_{0\text{-}120}$ of TRH (●) and Lau-TRH (○) following administration into the small intestine. Symbols represent the mean ± s.e. (n = 4–5).

percent of TRH) in homogenate of the rat small intestinal mucosa was approximately 100%, suggesting that TRH, the degradation product of Lau-TRH, was fairly stable in the intestinal mucosa for 60 min.

Discussion

We have previously reported the synthesis of Lau-TRH, a novel derivative of TRH, by chemical attachment of lauric acid to the *N*-terminal pyroglutamyl group of TRH; the central nervous system activity and endocrine activity of Lau-TRH were only slightly reduced to 81 and 64% of that of the parent TRH, respectively (Muranishi et al 1991). This finding suggested that the potency of TRH was little affected by the acylation of *N*-terminal pyroglutamyl residue of TRH by lauric acid, as Lau-TRH still possessed considerable TRH activity. Therefore, Lau-TRH is not a prodrug of TRH, as prodrugs are considered to have no pharmacological activity and only elicit activity after conversion to the parent drug.

In this study, a significant increase in plasma radioactivity of Lau-TRH following intestinal administration was observed compared with that of TRH. This result was mainly due to its increased lipophilicity compared with TRH as assessed by HPLC and partition coefficient experiments (Muranishi et al 1991). The partition coefficients of Lau-TRH were 1·91 and 2·14 in *n*-octanol buffer and $CHCl_3$ buffer, respectively, whereas those of TRH were 0·068 and 0·088, respectively. In HPLC analysis, the retention time of Lau-TRH (21·3 min) was delayed compared with that of TRH (8·01 min).

One problem with the present experiments was that we could not distinguish between the intact peptides and their metabolites in plasma. However, it was concluded that TRH and Lau-TRH were transported mainly in the intact forms, as TRH was relatively stable and Lau-TRH was degraded and converted to the native TRH only in the intestinal mucosa (Fig. 6). Therefore, the higher plasma radioactivity following administration of Lau-TRH is probably due to its high permeability across the intestinal mucosa. Another possible mechanism of enhancing Lau-TRH transport is by conversion to lauric acid, a degradation product of Lau-TRH, which can enhance the permeability of TRH in the gut.

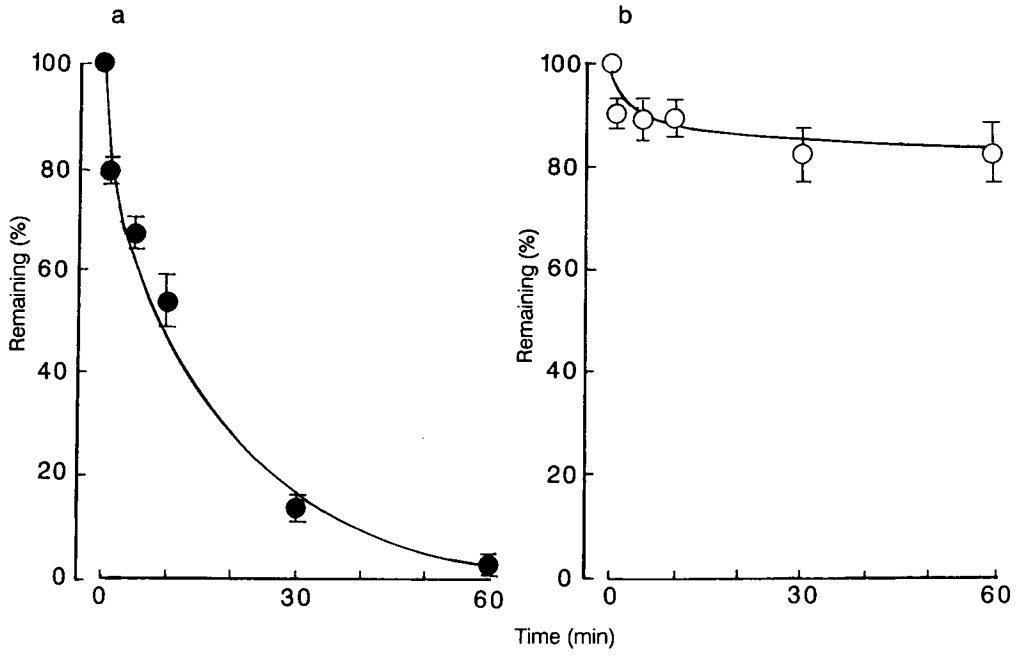

FIG. 5. Time course of degradation of TRH (a, ●) and Lau-TRH (b, ○) in rat plasma (37°C). Initial concentration: 0·1 mM. Symbols represent the mean ± s.e. (n = 3).

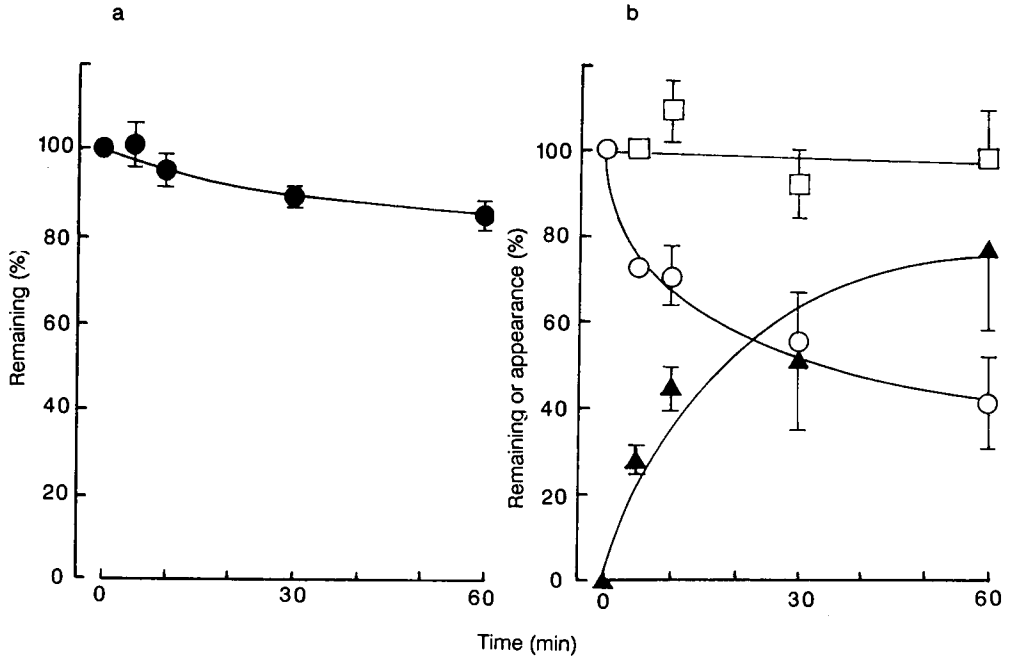

FIG. 6. Time course of degradation of TRH and Lau-TRH in the homogenates of the small intestinal mucosa (37°C). a. Remaining percent of TRH (●). b. Remaining percent of Lau-TRH (○), appearance percent of TRH (▲), remaining percent of Lau-TRH + appearance of TRH (□). Initial concentration: 0·5 mM. Symbols represent the mean ± s.e. (n = 3).

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.

Previous studies demonstrated that intestinal absorption of TRH was limited to the upper part of the intestine and was saturable in rats and dogs (Yokohama et al 1984a, b). In addition, the intestinal absorption of TRH was inhibited by the presence of oligopeptides and some β-lactam antibiotics which are known to be transported by active transport or carrier-mediated transport systems (Yokohama et al 1984b). From these results, it was suggested that TRH is transported

by a carrier-mediated transport system. The results of these reports were consistent with our present results that a dose-dependent plasma radioactivity expressed as percent of TRH was noted following administration into the small intestine (Fig. 2). Unlike TRH, we could not find a dose-dependent decrease in plasma radioactivity of Lau-TRH following administration into the small intestine (Fig. 3), suggesting that the absorption mechanism of Lau-TRH may be different from that of TRH. Similarly, Yokohama et al (1984c) reported that γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417), a new derivative of TRH, was absorbed in all parts of the small intestine and in a non-saturable manner, suggesting that DN-1417 is absorbed by a simple diffusion mechanism. From these results, it was suggested that chemical modification of TRH may decrease the affinity to the carrier protein of TRH.

In the stability experiments, the present study demonstrated that the degradation of TRH was very rapid and most of the TRH was hydrolysed in rat plasma for 60 min (Fig 6). This result was in agreement with the data of Møss & Bundgaard (1990b) who reported that the degradation of TRH showed first order kinetics with a degradation half-life of 9·4 min at low substrate concentration in human plasma. They also found that the initial step in the plasma-catalysed degradation of TRH was due to hydrolysis of the pyroglutamyl-histidyl bond by the TRH-specific pyroglutamyl aminopeptidase serum enzyme, resulting in the exclusive formation of histidyl-proline amide (His-Pro-NH_2), although we could not detect the metabolites of TRH in rat plasma.

Lau-TRH was much more stable than TRH in rat plasma (Fig. 5), suggesting that chemical modification of TRH by lauric acid might protect the degradation of TRH by TRH-specific serum enzymes such as pyroglutamyl aminopeptidase. Similar results were also reported by Bundgaard & Møss (1990) who demonstrated that *N*-alkoxycarbonyl prodrug derivatives of TRH were resistant to cleavage by the enzyme in plasma compared with TRH. These findings suggested that chemical modification of TRH might increase the plasma radioactivity by not only increasing the lipophilicity of TRH but also inhibiting the degradation of TRH.

In conclusion, the present study indicates that by chemical modification of TRH with lauric acid, it may be feasible to increase the lipophilicity and protect the degradation by TRH-specific enzymes, hence improving the intestinal absorption of this peptide. Undoubtedly, this chemical approach may be more applicable to various non-lipophilic peptides for systemic delivery rather than absorption promoters and protease inhibitors, since these modified peptides can specifically enhance their absorption without membrane damage and local irritation.

References

Banga, A. K., Chien, Y. W. (1988) Systemic delivery of therapeutic peptides and proteins. Int. J. Pharm. 48: 15–50

Bundgaard, H., Møss, J. (1990) Prodrugs of peptides. 6. Bioreversible derivatives of thyrotropin-releasing hormone (TRH) with increased lipophilicity and resistance to cleavage by the TRH-specific serum enzyme. Pharm. Res. 7: 885–892

Davis, S. S. (1989) Gastrointestinal absorption of polypeptides. In: Marshak, D., Liu, D. (eds) Current Communications in Molecular Biology: Therapeutic Peptides and Proteins; Formulations, Delivery and Targeting. Cold Spring Harbor Laboratory, USA, pp 41–45

Greenwood, F. C., Hunter, W. M., Glover, J. S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89: 114–123

Griffiths, E. C. (1985) Thyrotropin releasing hormone: endocrine and central effect. Psychoneuroendocrinology 10: 225–235

Hashida, N., Murakami, M., Yoshikawa, H., Takada, K., Muranishi, S. (1984) Intestinal absorption of carboxyfluorescein entrapped in liposomes in comparison with its administration with lipid-surfactant mixed miselles. J. Pharmacobiodyn. 7: 195–203

Hashimoto, M., Takada, K., Kiso, Y., Muranishi, S. (1989) Synthesis of palmitoyl derivatives of insulin and their biological activities. Pharm. Res. 6: 171–176

Hashizume, M., Douen, T., Muranishi, M., Yamamoto, A., Takado, K., Muranishi, S. (1992) Improvement of large intestinal absorption of insulin by chemical modification with palmitre acid in rats. J. Pharm. Pharmacol. 44: 555–559

Hunter, W. H., Greenwood, F. C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194: 495–496

Lee, V. H. L. (1986) Peptide and protein drug delivery: opportunities and challenges. Pharm. Int. 7: 208–212

Lee, V. H. L., Yamamoto, A. (1990) Penetration and enzymatic barriers to peptide and protein absorption. Adv. Drug Del. Rev. 4: 171–207

Møss, J., Bundgaard, H. (1990a) Prodrugs of peptides. 7. Transdermal delivery of thyrotropin-releasing hormone (TRH) via prodrugs. Int. J. Pharm. 66: 39–45

Møss, J., Bundgaard, H. (1990b) Kinetics and pattern of degradation of thyrotropin-releasing hormone (TRH) in human plasma. Pharm. Res. 7: 751–755

Møss, J., Buur, A., Bundgaard, H. (1990) Prodrugs of peptides. 8. In vitro study of intestinal metabolism and penetration of thyrotropin-releasing hormone (TRH) and its prodrugs. Int. J. Pharm. 66: 183–191

Muranishi, S., Sakai, A., Yamada K., Murakami, M., Takada, K., Kiso, Y. (1991) Lipophilic peptides: synthesis of lauroyl thyrotropin-releasing hormone and its biological activity. Pharm. Res. 8: 649–652

Yokohama, S., Yamashita, K., Toguchi, H., Takeuchi, J., Kitamori, N. (1984a) Absorption of thyrotropin-releasing hormone after oral administration of TRH tartrate monohydrate in the rat, dog and human., J. Pharmacobiodyn. 7: 101–111

Yokohama, S., Yoshioka, T., Yamashita, K., Kitamori, N. (1984b) Intestinal absorption mechanisms of thyrotropin-releasing hormone. Ibid. 7: 445–451

Yokohama, S., Yoshioka, T., Kitamori, N. (1984c) Absorption of γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417), an analog of thyrotropin-releasing hormone, in rats and dogs. Ibid. 7: 527–535