Development of New Lipophilic Derivatives of Tetragastrin: Physicochemical Characteristics and Intestinal Absorption of Acyl-tetragastrin Derivatives in Rats

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In order to improve the intestinal absorption of tetragastrin (TG), we synthesized lipophilic derivatives of TG by acylation of its N-terminal amino group with acetic acid, caproic acid, and lauric acid. The purified TG derivatives, acetyl-tetragastrin (Ac-TG), caproyl-tetragastrin (Cap-TG), and lauroyl-tetragastrin (Lau-TG), were confirmed to be more lipophilic than the parent TG by high-performance liquid chromatography (HPLC). The pharmacological activities and the intestinal absorption of TG and its derivatives were examined by measuring gastric acid secretion. Stimulation of gastric acid secretion by these derivatives after intravenous administration was stronger than with native TG. When the acetyl- and caproyl-derivatives were administered into the large intestinal loops, a marked increase in gastric acid secretion was observed in comparison with TG, while no significant effect occurred following administration of the TG derivatives into the small intestines. These results indicated that chemical modification of TG with fatty acids improves the absorption of TG from the large intestines.

KEY WORDS: tetragastrin; intestinal absorption; fatty acids; chemical modification; gastric acid secretion.

INTRODUCTION

Oral administration of peptides and proteins is often limited by their instability in the gastrointestinal environment and/or poor absorption from the gut (1). In order to promote the absorption of these drugs and reduce their degradation in the gut, various approaches have been examined. The use of absorption promoters and protease inhibitors has been shown to improve their intestinal absorption (2,3). However, limitations such as local irritation of the mucosa and nonselective absorption of other antigenic compounds are considered drawbacks in the use of these additives. Consequently, alternative methods are needed to improve the absorption of these peptides via the gastrointestinal tract.

Chemical modification can serve to improve the metabolic stability and intestinal absorption of peptides and proteins (4,5). These chemical methods aim at the modification of susceptible positions in the molecule while maintaining structural features essential for pharmacological activity. Rapid enzymatic inactivation and poor membrane penetration characteristics of thyrotropin releasing hormone (TRH) and insulin were overcome by chemical modification with fatty acids (6–10). Tetragastrin is the smallest part of the gastrin molecule (4C-terminal amino acids), which qualitatively shows the same physiological effects as gastrin (11). Like insulin and TRH, the intestinal absorption of this tetrapeptide was relatively poor because of its extensive hydrolysis in the gastrointestinal mucosa (12–14). We have synthesized new lipophilic derivatives of TG and determined whether these derivatives of TG possess the pharmacological activity of TG. We also describe whether these derivatives improve the absorption of TG in the small and large intestine.

MATERIALS AND METHODS

Materials

TG was purchased from Peptide Institute, Inc. (Osaka, Japan) and *N*-hydroxysuccinimide (HOSu), *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB), and *N,N'*-dicyclohexylcarbodiimide (DCC) were obtained from Watanabe Chemical Industry (Hiroshima, Japan). Lauric acid, caproic anhydride, acetic anhydride, dimethylformamide (DMF), and dimethylacetoamide (DMAc) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Trifluoroacetic acid (TFA), *N*-methylmorpholine, and all other chemicals used were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) and were of reagent grade.

Synthesis of TG Derivatives

New lipophilic derivatives of TG were synthesized by chemical attachment to N-terminal amino group with various fatty acids. The chemical structures of these compounds are shown in Fig. 1.

Acetyl-TG (AC-TG)

TG (24.11 mg) and *N*-methylmorpholine (8.15 μL) were dissolved in 1 mL of DMF. This solution was stirred with additional acetic anhydride (4.27 μL) at room temperature for 1 hr. The afforded solid material was recrystallized from dry ether (81.2% total yield).

Caproyl-TG (Cap-TG)

TG (24.33 mg) and *N*-methylmorpholine (3.71 μL) were dissolved in 1 mL of DMF. This solution was stirred with additional caproic anhydride (9.51 μL) at room temperature for 1.5 hr. The afforded solid material was recrystallized from dry ether (85.4% total yield).

Lauroyl-TG (Lau-TG)

Lau-OSu, synthesized by the method of Lapidot *et al.* (15), TG (24.11 mg), and *N*-methylmorpholine (8.15 μL) were dissolved in 1 mL of DMF. The solution was stirred with additional Lau-OSu (33.03-mg total) at room temperature for 3 hr. The afforded solid material was recrystallized from dry ether (80.0% total yield).

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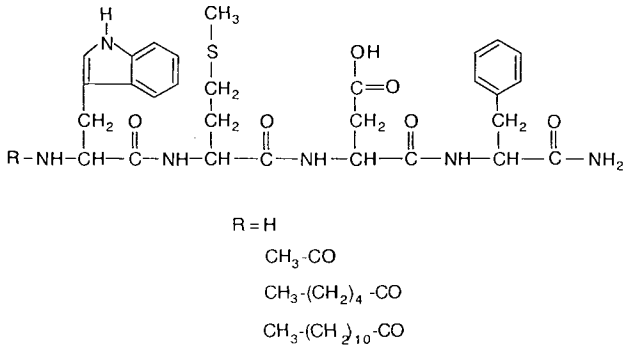

R = H
CH_3-CO
CH_3-$(CH_2)_4$-CO
CH_3-$(CH_2)_{10}$-CO

Fig. 1. Chemical structures of TG and its derivatives.

Physicochemical Characteristics of TG Derivatives

Melting Points

The melting points of TG and its derivatives were determined by a micro melting point apparatus (Yanagimoto Seisakusho, Kyoto, Japan) and are uncorrected.

Amino Acid Composition

The amino acid composition of TG and its derivatives was determined with Hitachi L-8500 amino acid analyzer. Acid hydrolysis was performed in evacuated sealed glass tubes with 6 *N* HCl at 110°C for 24 hr, and determinations of amino acid composition were performed with hydrolysate samples.

Determination of Lipophilicity

Retention Time on HPLC

TG and its derivatives were analyzed by reversed-phase HPLC (Hitachi) on a column (4.6 × 150 mm) of YMC-AM302 (ODS) (Fig. 3). The column was eluted with a linear gradient of acetonitrile (20–100%, 30 min) in 0.1% TFA at a flow rate of 0.7 mL/min. The eluate was monitored with a UV detector at a wavelength of 230 nm. The log k' values of TG and its derivatives were calculated using this formula.

$$\log k' = \log (t_r - t_{zero})/t_{zero}$$

where t_r is the retention time of TG and its derivatives and t_{zero} is that of solvent (acetonitrile:acetic acid = 1:1).

Pharmacological Activities of TG and Its Derivatives

The pharmacological activities of TG and its derivatives following intravenous administration were examined by activity of gastric acid secretion according to the method of Ghosh and Schild (Fig 2.) (16). Male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan), weighing 230–270 g at 7 weeks of age, were fasted for 48 hr before the experiments (but given water ad libitum). Under urethane anesthesia (1.5 g/kg i.p.), rats were surgically prepared for gastric perfusion. The stomach was rinsed and continuously perfused with 0.9% NaCl solution, and the perfusion rate was 1 mL/min using a peristaltic pump. TG or its derivatives was dissolved in 200 μL of phosphate-buffered saline (pH 7.4) containing 30% DMAc to yield a final concentration of 63.9 μ*M*. Dosing solutions (200 μL) were administered intravenously and the increase in gastric acid secretion was determined. The amount of gastric acid secretion into stomach perfusate was determined by a pH stat (titrant; 0.1 *N* NaOH). A control experiment was performed in which the basal gastric acid secretion was measured for 2 hr after intravenous administration of saline solution. In order to get the net effect in gastric acid secretion, the basal values were subtracted from the time–effect curves obtained. The increase in total acid output over the whole duration of TG and its derivatives activities were summed up following intravenous administration (area under time–effect curve minus basal secretion).

Intestinal Absorption of TG and Its Derivatives

Absorption experiments were performed by an *in situ* closed-loop method (9,10). Male Wistar strain rats (Japan SLC, Hamamatsu, Japan), 230–270 g, were fasted for 48 hr before the absorption experiments and then were anesthetized with urethane (1.5 g/kg, i.p.). The intestine was exposed through a midline abdominal incision. A small intestinal loop was prepared by cannulation with silicone tubing (i.d., 3 mm; o.d., 5 mm) at the proximal and distal ends of the small intestine. For the large intestine, an intestinal loop was prepared by cannulation with 3-cm silicone tubing (i.d., 3 mm; o.d., 5 mm) at the proximal and distal ends of the large intestine (approx. 8 cm long). TG or its derivatives were dissolved in 2 mL of phosphate-buffered saline (pH 7.4) containing 5% of DMAc to yield a final concentration of 3.21 m*M*. The drug solution (2 mL) was warmed to 37°C and placed into the small or large intestinal loop, which was closed by clipping with forceps at the cannulated position of each tubing. An increase in acid output was determined for evaluating the absorption of TG and its derivatives as mentioned above.

Statistical Analyses

Results are expressed as the mean ± SE and statistical significance was performed using a Student's *t* test.

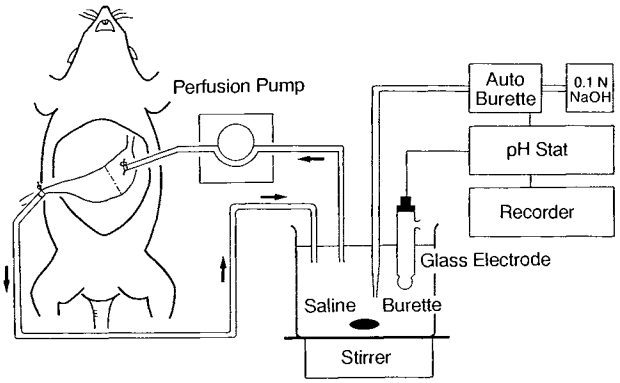

Fig. 2. Apparatus for the determination of gastric acid output following intravenous or intestinal administration of TG and its derivatives.

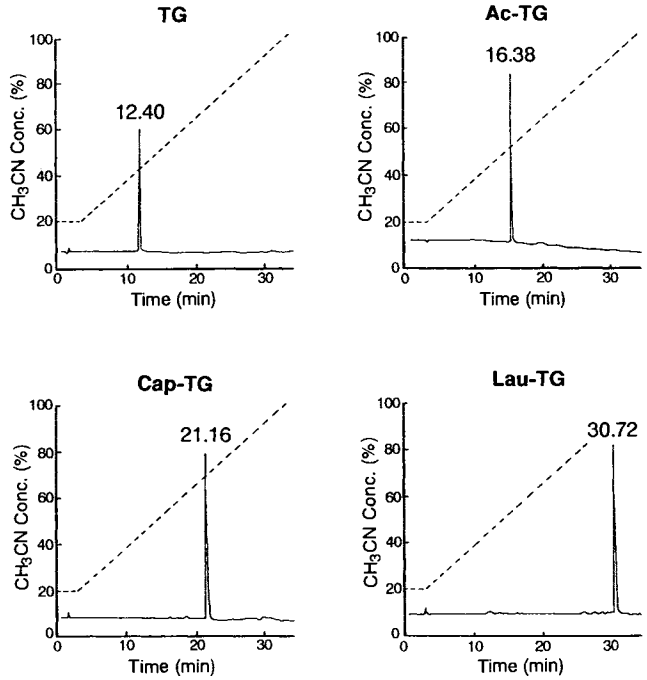

Fig. 3. HPLC chromatograms of TG and its derivatives. The HPLC conditions are as follows: flow rate, 0.7 mL/min; column, YMC AM-302 (4.6 × 150-mm, ODS); mobile phase A, 0.1% trifluoroacetic acid; mobile phase B, acetonitrile; detection wavelength, 230 nm. (- - -) HPLC gradient concentration of acetonitrile.

RESULTS

Chemistry

The synthetic derivatives of TG were purified and isolated by preparative HPLC. Figure 3 shows HPLC analyses of TG and its derivatives. These purified and isolated compounds showed single peaks at different positions than native TG on HPLC, indicating that the acyl derivatives were homogeneous compounds. The retention times were ranked as follows: Lau-TG (30.7 min) > Cap-TG (21.2 min) < Ac-TG (16.4 min) > TG (12.4 min). The logk' values of these compounds were also calculated and are listed in Table I. The logk' value increased with increasing carbon number of fatty acids attached to TG, indicating that the acyl derivatives enhanced the lipophilicity of TG. Table I also shows amino acid ratios and melting points of these compounds. The amino acid ratio of the derivatives were similar to that of native TG, suggesting that they were not degraded during synthesis and possessed the structure of native TG.

Table I. Amino Acid Ratio (Phe = 1.00), Melting Point, and log k' of TG and Its Derivatives

	Amino acid ratio[a]	Melting point (°C)	logk'
TG			
Trp	—		
Met	—	204–206	0.6925
Asp	—		
Ac-TG			
Trp	0.89		
Met	0.90	220–222	0.8023
Asp	0.99		
Cap-TG			
Trp	0.87		
Met	0.91	228–230	0.9127
Asp	1.00		
Lau-TG			
Trp	0.85		
Met	0.85	242–243	1.0585
Asp	1.00		

[a] Amino acid ratio of hydrolysate of TG and its derivatives in 4*N*-methane sulfonic acid containing tryptamine.

Table II. Pharmacological Activities of TG and Its Derivatives After Intravenous Administration

	Increase in total acid output (μEq)[a]	Ratio
TG	20.76 ± 3.91	1.00
Ac-TG	36.13 ± 8.10[b]	1.74
Cap-TG	35.93 ± 6.90[b]	1.73
Lau-TG	32.35 ± 8.46[b]	1.56

[a] Each value represents mean ± SE (n = 4–6).
[b] Not significantly different, compared with TG.

Pharmacological Activities of TG and Its Derivatives

Table II shows an increase in total acid output after intravenous administration of TG and its derivatives. The derivatives of TG appeared to be more effective than native TG after intravenous administration, although differences were not statistically significant. The efficacy of acetyl-, caproyl-, and lauroyl-derivatives was 174, 173, and 156%, respectively, relative to TG. These findings indicate that acylation of TG did not reduce an activity of gastric acid output of TG.

Intestinal Absorption of TG and Its Derivatives

Figures 4 and 5 show acid output–time curves after small or large intestinal administration of TG and its derivatives. There was a significant increase in total acid output following large intestinal administration of Ac-TG and Cap-TG in comparison with TG, while we found no significant difference between TG and Lau-TG following large intestinal

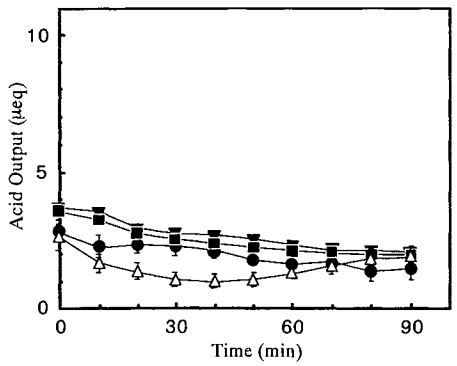

Fig. 4. Acid output–time curves after small intestinal administration of tetragastrin and its derivatives in DMAc solution. Results are expressed as the mean ± SE of five rats. (△) TG; (■) Ac-TG; (●) Cap-TG; (▼) Lau-TG.

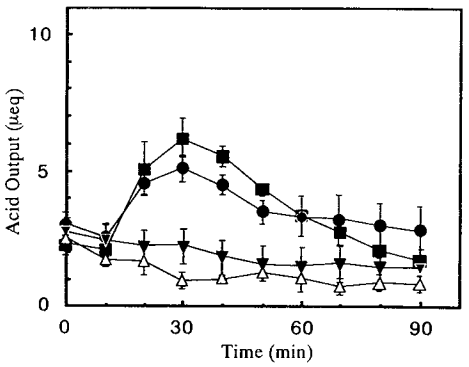

Fig. 5. Acid output–time curves after large intestinal administration of tetragastrin and its derivatives in DMAc solution. Results are expressed as the mean ± SE of five rats. (△) TG; (■) Ac-TG; (●) Cap-TG; (▼) Lau-TG.

administration (Fig. 5). In contrast, no significant increase in gastric acid output was obtained following small intestinal administration of these derivatives (Fig. 4). Table III summarizes the increase in total acid output after small or large intestinal administration of TG and its derivatives in DMAc solution. As is evident from the table, the amounts of total acid output after large intestinal administration of Ac-TG and Cap-TG were 74.3–133 times greater than native TG. In contrast, there was no significant increase in acid output following small intestinal administration of these derivatives in comparison with TG.

DISCUSSION

Tetragastrin is a C-terminal tetrapeptide amide, Try-Met-Asp-Phe-NH_2, of gastrin with all the physiological effects of the parent molecule, although it is not as potent as gastrin heptadecapeptide on a molar basis (11,14). Removal of the C-terminal amide group to give the tetrapeptide free acid causes loss of acid stimulating activity and the amide group on the C-terminal phenylalanine is essential for gastric stimulation and other hormonal action.

We have synthesized acyl-TG analogues that maintain the pharmacological activity of TG. This result agrees with a previous report that lauroyl-TRH (lauric acid-TRH conjugate) maintains 64–81% of TRH activity (7). Similar results were obtained in the case of insulin derivatives (6). The reason these TG analogues appeared to be more potent than the native TG is not fully understood. In pilot studies, these TG derivatives were more stable than the native TG in plasma and various intestinal mucosal homogenates. The high stability of acyl-TG derivatives in plasma may be related to the high activity of these compounds, since tetragastrin can be digested by the enzymes in the pancreatic and entire juice by mucosal enzymes, including those of the brush border, and by enzymes in the blood and, especially, in the liver, before reaching the receptor site in the stomach (12). Alternatively, the acyl derivatives may have a higher affinity for the receptor site than the native TG.

Table III. Total Acid Output After Small and Large Intestinal Administration of TG and Its Derivatives[a]

	Small intestine (μEq)	Large intestine (μEq)
TG	0.18 ± 0.18	0.10 ± 0.05
Ac-TG	0	13.3 ± 2.13**
Cap-TG	1.49 ± 1.16	7.43 ± 2.57*
Lau-TG	0.12 ± 0.08	0.27 ± 0.18

[a] Each value represents mean ± SE (n = 4–6).
* $P < 0.05$, compared with TG.
** $P < 0.001$, compared with TG.

We evaluated the intestinal absorption of TG and its derivatives by measuring acid output activity instead of plasma concentrations of these drugs. This method can be used as a good index for determining intestinal absorption, since a linear correlation exists between the logarithm of the intravenous dose and the acid output activities over the range of 0–5 mM for all compounds studied (data not shown).

The gastric acid secretion activities of Cap-TG and Lau-TG were higher than that of the native TG following large intestinal administration, while no marked increase in the acid secretion activities occurred after small intestinal administration of these derivatives (Figs. 4 and 5, Table III). These differences might be accounted for by the stability of the derivatives in the gastrointestinal lumen. In general, the enzyme activities responsible for the hydrolysis of peptides and proteins in the small intestine were higher than in the large intestine. Indeed, the half-life for the degradation of Cap-TG in homogenate of the small intestinal mucosa was much shorter than that in the large intestine ($t_{1/2}$ = 5.75 ± 0.69 for small intestine; $t_{1/2}$ = 63.0 ± 11.1 for large intestine). Similar results were also obtained in the case of Ac-TG. Consequently, since TG and its derivatives are highly degraded in the small intestine before reaching the systemic circulation, stimulation of acid output activities was nondetectable following administration into the small intestine. In contrast, the marked increase in acid output activities following large intestinal administration of the TG derivatives may be due to their stability in the large intestine.

Unexpectedly, we found no significant difference between TG and Lau-TG following large intestinal administration, although the lipophilicity of Lau-TG was much higher than that of native TG. These results suggested that a optimal lipophilicity exists for improving the large intestinal absorption of TG. Lau-TG, a highly lipophilic derivative, may not be easily transported across the unstirred water layer of the large intestinal lumen and it may accumulate in the intestinal tissue, resulting in decreased transport across the large intestinal membrane. However, it is unlikely that Lau-TG was totally degraded in the intestinal lumen before reaching the systemic circulation, since Lau-TG was more stable than Ac-TG and Cap-TG in homogenates of the small and large intestine in our pilot studies.

In conclusion, the present study demonstrated that absorption of TG from the large intestine was enhanced by acylation with certain fatty acids. This chemical modification approach may be useful for improving the large intestinal absorption of TG.

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