J. Pharm. Pharmacol. 1992, 44: 555-559
Received September 10, 1991

© 1992 J. Pharm. Pharmacol.

Improvement of Large Intestinal Absorption of Insulin by Chemical Modification with Palmitic Acid in Rats

MINORU HASHIZUME, TAKAYUKI DOUEN, MASAHIRO MURAKAMI, AKIRA YAMAMOTO, KANJI TAKADA AND SHOZO MURANISHI

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

Abstract—The intestinal absorption of ^{125}I-labelled palmitoyl insulin was examined following administration into in-situ closed large intestinal loops of rats. When mono- and dipalmitoyl insulins (Palins-1 and Palins-2, respectively) were administered in polyoxyethylene hydrogenated castor oil (HCO 60) micellar system into intestinal loops, a marked increase in plasma radioactivity and a corresponding disappearance of residual radioactivity in the intestinal lumen were observed in the following rank order: Palins-2 > Palins-1 > native insulin. In addition, the derivatives were more stable than native insulin in the mucosal tissue homogenates of the large intestine. These results suggest that chemical modification of insulin with palmitic acid may not only increase the lipophilicity of insulin but also reduce its degradation, resulting in the increased transfer of insulin across the large intestinal mucous membrane. The linoleic acid-HCO 60 mixed micelles system did not have a significant effect on the large intestinal absorption of radioactivity associated with the lipophilic insulin analogues.

Although several investigators have demonstrated the uptake of peptides and proteins from the intestine into the systemic circulation, only small amounts of intact molecules are absorbed by mature animals (Bloch et al 1988; Shichiri et al 1972). This poor absorption characteristic is partly explained by the low stability of the peptides and proteins to the acid environment of the stomach and digestive enzymes present in the lumen or brush-border of the intestine (Lee & Yamamoto 1989). Consequently, they usually do not survive transport through these gastrointestinal barriers. Another reason for their low bioavailability is their poor permeability characteristics due to their low lipophilicity and large molecular size.

Various approaches have been examined to overcome the delivery problems of these peptides and to improve their absorption via the gastrointestinal tract. Of these approaches, the use of absorption enhancers has been shown to improve the small intestinal and colonic absorption of macromolecules containing polypeptides (Muranishi 1990). However, limitations such as local irritation of the mucosa and nonselective absorption of other antigenic compounds are considered drawbacks in the use of absorption enhancers. Therefore, alternative methods are needed for peptide delivery via the gastrointestinal tract.

A potential useful approach to solve these delivery problems may be chemical modification of peptides and proteins to produce prodrugs and analogues. Thus, it is plausible that this chemical approach may protect peptides against degradation by peptidases and other enzymes present at the mucosal barrier and renders the peptides and proteins more lipophilic, resulting in increased bioavailability. From these standpoints, we have already synthesized a novel lipophilic derivative of thyrotropin-releasing hormone (TRH) by chemical attachment of lauric acid to the *N*-terminal pyroglutamyl group of TRH (Muranishi et al 1991), since the importance of lipid solubility in gastrointestinal absorption has long been recognized and this was exemplified by a good bioavailability of cyclosporin A, a lipophilic peptide (Takada et al 1986). Our previous studies of TRH demonstrated that this new derivative of TRH increased the lipophilicity and protected against degradation by the TRH-specific enzymes, hence improving the intestinal absorption of the peptide.

Furthermore, in a previous study, we have also synthesized novel lipophilic insulins by palmitoylation of native insulin (Hashimoto et al 1989). Two purified derivatives, B1-monopalmitoyl and B1, B29-dipalmityl insulin (Palins-1 and Palins-2, respectively), with biological activity were obtained. In the present work, the intestinal absorption of synthetic insulin derivatives and native insulin were compared using ^{125}I-labelled compound.

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

Materials and Methods

Materials

Crystalline bovine insulin (25·6 int. units mg^{-1}) was purchased from Sigma Chemical Company (St Louis, MO, USA). Palins-1 and Palins-2 were synthesized as described previously (Hashimoto et al 1989). [^{125}I]Iodine was purchased from Daiichi Pure Chemicals Co. Ltd (Tokyo, Japan) as a solution obtained by distilling $Na^{125}I$ into 10^{-5} M NaOH at the highest available concentration. Linoleic acid of high purity (>99%) was kindly supplied by Nippon Oil & Fats Co. Ltd (Tokyo, Japan). Polyoxyethylene hydrogenated castor oil (HCO 60) was also supplied by Nikko Chemicals Co. Ltd (Tokyo, Japan). All other chemicals used were of reagent grade.

Method of ^{125}I-labelling and purification

The method of iodination was based on a modification of McFarlane's iodine monochloride (Springell 1961; Glover et al 1967). A 37·7 μL sample of diluted iodine monochloride

(containing 11·7 μg of iodine) and 50 μg of native insulin or palmitoyl insulins in 20 μL of acetate buffer (pH 2·8) were added to 35 μL of diluted [^{125}I]iodine solution (600 μCi) in a glass tube (8 × 55 mm). The reaction was carried out for 5 min at room temperature (21°C) and stopped by the addition of 0·1 M $Na_2S_2O_3$ solution. The resultant mixture was subjected to gel filtration through a Sephadex G-25 fine column (1·2 × 23 cm) to remove free iodine. The identification of the iodinated native insulin and palmitoyl insulins was performed by TLC. The specific activities of iodinated insulin, Palins-1 and Palins-2 were 6·22, 5·48 and 6·24 μCi μg^{-1}, respectively. One mol of native insulin and its derivatives bound approximately 8 mol ^{125}I. The iodinated native insulin and palmitoyl insulins were used for absorption and stability experiments immediately after the iodination.

Preparation of HCO 60 solutions

A test solution was prepared by adding the labelled native insulin or palmitoyl insulins solutions to 2·7 mM HCO 60 (pH 7·4) isotonic phosphate-buffered solution (PBS). The pH of the resultant solution was adjusted to 6·7 with sodium bicarbonate.

Preparation of linoleic acid-HCO 60 mixed micelles (LMM)

A solution of LMM was prepared by dispersing linoleic acid and HCO 60 in pH 7·4 PBS, followed by sonication with an Ohtake model 5202 sonicator (Ohtake Seisakusho Co. Ltd, Tokyo, Japan) in an ice-water bath 50 W for 5 min (Fukui et al 1987). This solution was mixed with the labelled native and palmitoyl insulins with a Vortex mixer. The concentrations of linoleic acid and HCO 60 used for absorption experiments were 20 and 2·7 mM, respectively.

Absorption experiments

Absorption experiments were performed on 250–300 g male Wistar albino rats (Japan SLC, Inc., Hamamatsu, Japan) by an in-situ closed loop method (Hashida et al 1984). Animals were fasted for 18 h before the experiments, with water freely available, and anaesthetized with intraperitoneal sodium pentobarbitone (32 mg kg^{-1}) during the experiments. The intestine was exposed through a midline incision. An intestinal loop was prepared by cannulation with 3 cm silicon tubing (i.d., 3 mm: o.d., 5 mm) at the proximal and distal ends of the large intestine (approx. 8 cm long). The drug solution warmed at 37°C was introduced into the intestinal loop, which was closed by clipping with forceps at the cannulated position of each tubing. The administered volume was 3 mL for the large intestine (containing 0·17 μCi of radioactivity). Blood samples were collected from the jugular vein and the blood was centrifuged to obtain plasma. Plasma radioactivity was determined with a gamma counter (Aloka Auto Well Gamma System ACR-500).

The amount of radioactivity remaining in the large intestinal loops was determined as follows: the intestine was removed from the rat at selected times after dosing, and the residual radioactivity in the whole loop (the remaining solution and the intestinal tissue) was determined with a gamma counter. The intestinal tissue was then rinsed several times with 20 mL of saline (0·9% NaCl), and the radioactivity in the tissue alone was determined.

In-vitro stability with tissue homogenate supernatant

In-vitro stability experiments of insulin and its analogues were performed with the large intestine according to the method of Fujii et al (1985) with some modifications. The large intestines of rats were collected and washed with saline. The mucosal surface of the intestine was scraped off and collected. A 20% homogenate was prepared by homogenizing the mucosal surface in saline using a Polytron homogenizer (Kinematica GmbH, Switzerland). The homogenate was centrifuged at 10 000 *g* for 60 min and the supernatant was used for the stability experiments. In order to estimate the stability of the insulin derivatives in the large intestinal mucosal homogenate, the test solution was incubated in-vitro with the supernatant at 37°C for 60 min. After incubation, the mixture was rapidly cooled to 0°C, applied to a Sephadex G-25 column (0·6 × 25 cm), and eluted with pH 2·8 acetate buffer.

Results

Absorption of insulin and its derivatives from the large intestine

Fig. 1 shows the time course of plasma radioactivity following administration of 2·7 mM HCO 60 solution into the large intestinal loop. Appreciable amounts of radioactivity were detected for 6 h in the plasma after administration of Palins-1 and Palins-2, while only a slight amount of radioactivity was observed in the case of native insulin. The maximal radioactivity in plasma observed after administration of Palins-2 was 6-fold higher than after underivatized insulin. Palins-1-derived radioactivity was 3-fold higher than underivatized insulin.

The residual radioactivities in the large intestine were periodically determined. Fig. 2 shows the time course of the residual radioactivities in the whole loop and in the intestinal tissue. The residual activities gradually decreased during 6 h in all cases. Palins-2 showed the greatest decline. No obvious differences among the three test materials were found in their localization within large intestinal tissues. At 6 h, the residual radioactivities in the whole loop were 74·8% (native insulin), 67·1% (Palins-1) and 52·1% (Palins-2). Therefore, the rank

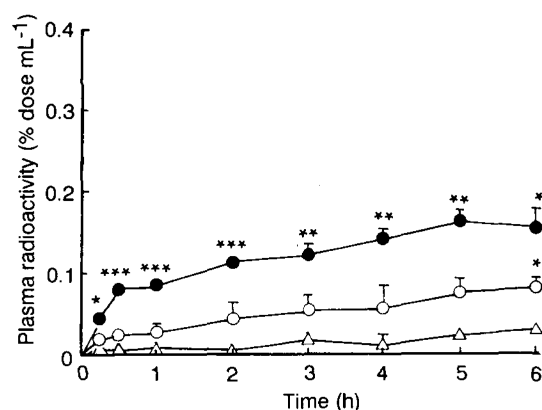

FIG. 1. Plasma radioactivity of insulin and its derivatives following administration of 2·7 mM HCO 60 solution into the large intestine. ○ Palins-1, ● Palins-2, △ native insulin (control). Each point represents mean ± s.e. of 3–6 experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from native insulin by Student's *t*-test.

Table 1. Comparison of stability of native insulin and palmitoyl insulins after incubation with large intestinal tissue homogenate.

Compound	Radioactivity of original compound (counts min^{-1})	Total radioactivity[a] (counts min^{-1})	Percentage as original compound[b]
Native insulin	15188 ± 1208·9	48053 ± 987·5	31·5 ± 1·8
Palins-1	33996 ± 1347·5	45157 ± 1156·4	75·2 ± 1·3
Palins-2	41218 ± 917·1	50248 ± 2103·6	82·2 ± 1·6

Each value represents the mean with standard error of a group of three. [a]Original compound and metabolites. [b]Percentage of original compound to total radioactivity.

order of disappearance of radioactivity from the large intestinal loop was similar to that observed in the plasma concentration experiment.

Stability of insulin and its derivatives in the large intestinal epithelium

The stability of insulin and its derivatives in the large intestinal epithelium was examined by incubating the supernatant of a large intestinal tissue homogenate for 60 min at 37°C. After incubation, gel filtration was performed using a Sephadex G-25 column for separation of the ^{125}I-labelled metabolic fragment. The elution profiles of native insulin, Palins-1 and Palins-2 after incubation for 60 min are shown in Fig. 3. Fraction numbers 8–13 correspond to the elution position of the labelled parent insulins, the radioactivity in fraction numbers 15–35 may represent metabolites of each of the labelled insulins and fraction 40 is the usual elution position of free ^{125}I. The sum of the radioactivity in the fractions and the total recovery of radioactivity is shown in Table 1.

Effect of LMM on the large intestinal absorption of insulin and its derivatives

The effect of LMM (20 mM linoleic acid and 2·7 mM HCO 60), a typical absorption enhancer, on the large intestinal absorption of the new insulin derivatives was also examined. Fig. 4 shows the plasma radioactivity following administration of these labelled compounds into the large intestinal loop. The plasma radioactivity associated with insulin was increased about tenfold by LMM. However, the promoting effect of LMM on the plasma radioactivity associated with palmitoyl insulins was found to be less pronounced; about twofold.

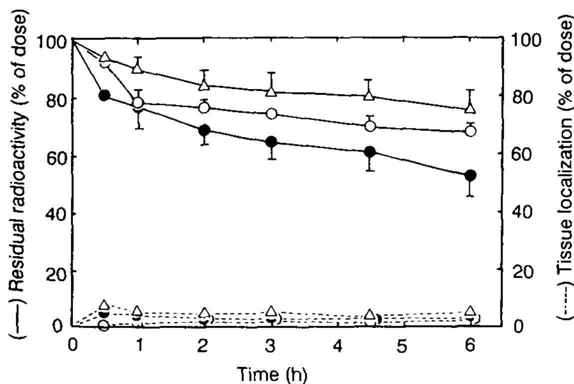

FIG. 2. Residual radioactivity of insulin and its derivatives in large intestinal loop and intestinal tissue. ○ Palins-1, ● Palins-2, △ native insulin. Each point represents the mean ± s.e. of 3–4 experiments.

Discussion

The present study demonstrated that a marked increase in plasma radioactivity was observed after the administration of Palins-1 and Palins-2 in comparison with native insulin. This result was in good agreement with the result that plasma radioactivity of Lau-TRH, a new lipophilic TRH derivative, significantly increased following the small intestinal administration compared with that of TRH (Muranishi et al 1991).

In addition, our previous study indicated that the lipophilicity of the insulin analogues was increased by palmitoylation (Palins-2 > Palins-1 > native insulin) as assessed by

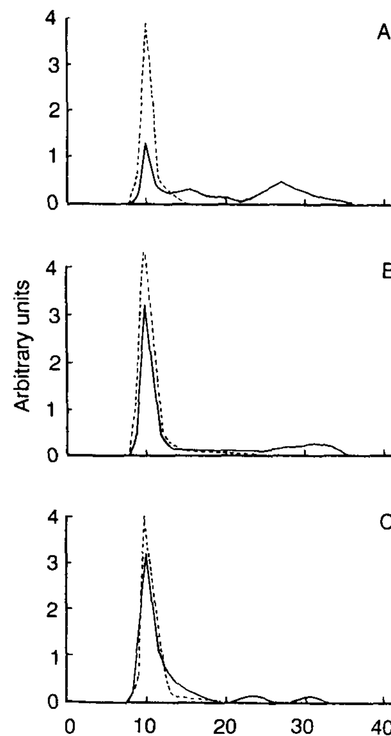

FIG. 3. Elution profile of native insulin and palmitoyl insulins obtained after incubation with a large intestinal epithelium homogenate for 60 min at 37°C and Sephadex G-25 gel permeation chromatography. A. Native insulin, B. Palins-1, C. Palins-2. Similar patterns were obtained from three different experiments. Test solution (- - -) and sample (——) after incubation with homogenate.

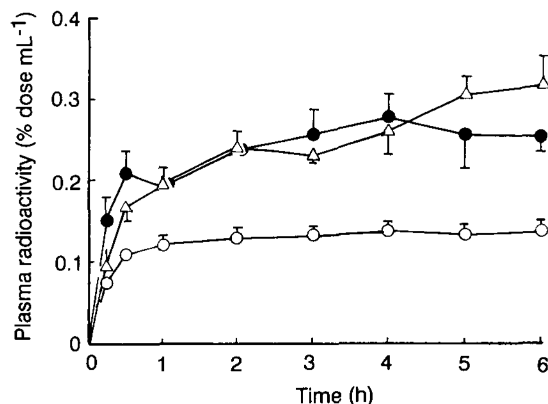

FIG. 4. Change in plasma radioactivity of insulin and its derivatives following administration of LMM (20 mM linoleic acid and 2·7 mM HCO 60) into the large intestine. ○ Palins-1, ● Palins-2, △ native insulin. Each point represents the mean ± s.e. of 3–6 experiments.

retention time on HPLC (Hashimoto et al 1989). Accordingly, these observations suggested that increased lipophilicity of insulin by palmitoylation may improve its intestinal absorption. This result was also supported by the experiment of residual radioactivity in the large intestinal lumen and tissue where the rank order of disappearance of radioactivity from the large intestinal loop was similar to that observed in the plasma concentration experiments (Fig. 2).

Another reason for increasing the plasma radioactivity following the large intestinal administration of Palins is the high stability of these new insulin derivatives. As shown in Fig. 3 and Table 1, these derivatives were more stable than native insulin in the mucosal tissue homogenate of the large intestine. This result suggests that chemical modification of insulin with palmitic acid may not only increase the lipophilicity of insulin but also reduce the degradation of insulin, resulting in the increased transfer of insulin across the large intestinal mucous membrane. Another possible mechanism for enhancing the absorption of Palins is that palmitic acid, which was the degradation product of Palins, may enhance the permeability of insulin in the large intestine. However, this mechanism is unlikely, since the concentration of this fatty acid was too low to elicit the absorption enhancing properties in the large intestine.

Kabanov et al (1989) reported that fatty acid proteins acquire an ability to translocate across lipid membranes and penetrate intact cells. They demonstrated a directed transport of fatty acylated $F(ab)_2$ and F(ab) fragments of antibodies against tissue specific antigens of brain glial cells across the hepatoencephalic barrier. They also showed that fatty acid antiviral antibodies, capable of crossing the biological membranes, were found to decrease drastically the virus reproduction in infected cells. Our results seem to be supported by those of Kabanov et al (1989). One problem of this experiment is that the determination of radioactivity in plasma may include metabolic fragments of insulin and its analogues. However, in our preliminary experiment, insulin and its new derivatives were stable in plasma as assessed by HPLC. The remaining percentages of insulin and Palins-1 in plasma at 3 h were $98{\cdot}8 \pm 4{\cdot}2$ and $90{\cdot}6 \pm 1{\cdot}9\%$ respectively. Furthermore, as shown in Fig. 3 and Table 1, lipid modification of insulin by chemical modification of the hydrophobic moiety may provide protection from enzymatic degradation at the administration site. Therefore, these metabolic experiments suggest that the increase in plasma concentration of palmitoyl insulin relative to insulin in the previous experiment (Fig. 1) is not attributable to a degradation product.

Recently, various absorption enhancers, such as surfactants, chelating agents, bile salts and fatty acids have been utilized to improve the intestinal absorption of poorly absorbed drugs (Muranishi 1990). Of the absorption enhancers, LMM is one of the most effective enhancers which is non-toxic to the intestinal mucosa. However, as demonstrated in Fig. 4, the LMM did not have a significant effect on the large intestinal absorption of these lipophilic insulin analogues. The reason why the effect of LMM on the intestinal absorption of insulin derivatives was less than native insulin may be the different absorption characteristics of these compounds. Kimura et al (1971) demonstrated that sodium cholate, one of the absorption enhancers, considerably promoted the intestinal absorption of poorly lipid soluble drugs and it was less effective in promoting the absorption of lipid soluble drugs; in some cases it inhibited their absorption. For lipid soluble drugs, the concentration of drug–micelle interactions may be important in their intestinal absorption (Kakemi et al 1965). Therefore, it may be considered that the promoting effect of LMM was much more pronounced on the absorption of poorly lipid soluble native insulin than lipophilic insulin derivatives.

In summary, the present findings suggest that chemical modification to improve the lipophilicity of insulin can cause an increase in the large intestinal absorption and the stability of insulin in the large intestinal lumen.

References

Bloch, K. J., Wright, J. A., Bishara, S. M., Bloch, M. B. (1988) Uptake of polypeptide fragments of proteins by rat intestine in vitro and in vivo. Gastroenterology 95: 1272–1278

Fujii, S., Yokoyama, T., Ikegaya, K., Sato, F., Yokoo, N. (1985) Promoting effect of the new chymotrypsin inhibitor FK-448 on the intestinal absorption of insulin in rats and dogs. J. Pharm. Pharmacol. 37: 545–549

Fukui, H., Murakami, M., Yoshikawa, H., Takada, K., Muranishi, S. (1987) Studies on the promoting effect of lipid-surfactant mixed micelles (MM) on intestinal absorption of colloidal particles. Dependence on particle size and administration site. J. Pharmacobiodyn. 10: 236–242

Glover, J. S., Salter, D. N., Shepherd, B. P. (1967) A study of some factors that influence the iodination of ox insulin. Biochem. J. 103: 120–128

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 micelles. J. Pharmacobiodyn. 7: 193–195

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

Kabanov, A. V., Levashov, A. V., Alakhov, V. Y. (1989) Lipid modification of proteins and their membrane transport. Protein Engineering 3: 39–42

Kakemi, K., Arita, T., Muranishi, S. (1965) Absorption and excretion of drugs. XXVII. Effect of nonionic surface-active agents on rectal absorption of sulfonamide. Chem. Pharm. Bull. 13: 976–985

Kimura, T., Inui, K., Sezaki, H. (1971) Effect of bile salts on the

gastrointestinal absorption of drugs III. Effect of sodium cholate on the intestinal absorption of sulfa drugs. Yakuzaigaku 31: 167–174

Lee, V. H. L., Yamamoto, A. (1989) Penetration and enzymatic barriers to peptide and protein absorption. Adv. Drug Del. Rev. 4: 171–207

Muranishi, S. (1990) Absorption enhancers. Crit. Rev. Therap. Drug Carrier Syst. 7: 1–33

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

Shichiri, M., Kawamori, R., Karasaki, K., Okada, A., Shigeta Y., Abe, H. (1972) Increase in plasma immunoreactive insulin following administration of insulin to the gastrointestinal tract of rabbits. Diabetes 21: 203–208

Springell, P. H. (1961) An unreactive tyrosine residue in insulin and the exclusive iodination of the A chain. Nature 191: 1372–1373

Takada, K., Yoshimura, H., Shibata, N., Masuda, Y., Yoshikawa, H., Muranishi, S., Yasumura, T., Oka, T. (1986) Effect of administration route on the selective lymphatic delivery of cyclosporin A by lipid-surfactant mixed micelles. J. Pharmacobiodyn. 9: 156–160