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In vivo effects of highly purified docosahexaenoic acid on rectal insulin absorption

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Abstract

The purpose of this study was to evaluate the effectiveness and the toxicity of polyunsaturated fatty acid, such as oleic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as a potential absorption enhancer for rectal delivery of insulin, using a water-in-oil-in-water (W/O/W) multiple emulsion. In a single administration study, rectal insulin absorption was enhanced markedly, and marked hypoglycemia was induced by the emulsion incorporating various fatty acids in an insulin dose-related fashion. The pharmacological availability of the emulsion incorporating 2% oleic acid, EPA and DHA was ≈ 7.7, 11.0 and 25.4%, respectively. The insulin absorption enhancement effect was not increased in proportion to the amount of DHA in the emulsion at $> 1\%$. In addition, by incorporating Pluronic F 127 (PF 127) into the emulsion, the mean T_{max} value of the serum glucose–time curve could be extended to twice that of the emulsion without PF 127. In a multiple administration study, the mean $AUC_{glucose}$ values of the emulsion incorporating DHA showed almost the same value on the first and the tenth day. From the morphological appearance of the mucosal surface, the emulsion incorporating DHA induced no or little mucosal damage. Our findings demonstrated that DHA has a strong insulin permeability enhancement effect and little toxicity. Thus, DHA is an attractive candidate as an absorption enhancer for intestinal delivery of insulin.

Keywords: Insulin; Emulsion; Docosahexaenoic acid; Pluronic F 127; Rectal absorption; Multiple administration

1. Introduction

Insulin generally exhibits poor bioavailability ($< 1\%$) on oral administration. The poor bioavailability has been attributed to the extensive hydrolysis of insulin by the proteolytic enzymes in the gastrointestinal (GI) tract and a poor membrane permeability because of high molecular weight. To overcome problems of absorption via the GI tract, various strategies, such as the use of absorption enhancers (Amino et al., 1988; Mor-

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ishita et al., 1993; Utoguchi et al., 1998) and protease inhibitors (Morishita et al., 1992; Yamamoto et al., 1994), dosage form (Takouchi et al., 1996; Tozaki et al., 1997) and chemical modification (Hashizume et al., 1992; Muranishi et al., 1992), have been tried. Water-in-oil-in-water (W/O/W) multiple emulsions were thought to be an effective dosage form as an enteral carrier of insulin because they could protect insulin against proteolysis (Cunha et al., 1997). In addition, various types of absorption enhancers can be used and incorporated in each phase, in accordance with its solubility. Therefore, we have studied whether it was possible to increase the intestinal absorption of insulin using W/O/W emulsion incorporating a range of absorption enhancers (Matsuzawa et al., 1995; Morishita et al., 1998; Suzuki et al., 1998).

We recently documented that long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5 ω3) and docosahexaenoic acid (DHA, 22:6 ω3) were effective intestinal absorption enhancers of insulin (Morishita et al., 1998; Suzuki et al., 1998) and vancomycin (Morishita et al., 1999). We also showed that their effects were stronger than those of C18 unsaturated fatty acids (Suzuki et al., 1998), such as oleic acid. EPA and DHA, extracted from fish oil, are essential polyunsaturated fatty acids highly enriched in brain lipids and retinal photoreceptor cell phospholipids. Recently, the influence of these polyunsaturated fatty acids on various biological membranes has been reported (Hawthorne et al., 1990; Stillwell et al., 1993). Stillwell et al. (1993) found that lipid bilayers which had been enriched with DHA are more permeable to several solutes than are non-modified membranes. From these findings and our previous studies, it is conceivable that DHA could be a candidate as a membrane permeability modifier in a drug delivery system.

Our previous in situ absorption study showed that insulin emulsions incorporating EPA or DHA could induce marked hypoglycemia when administered to the colon and the rectum (Suzuki et al., 1998). To clarify their effectiveness and toxicity in vivo, single and multiple rectal administration experiments were performed in the present study. In the single administration study, dose-related hypoglycemic effects of insulin emulsions were determined. Effects of DHA amount in the emulsion on insulin absorption were also examined. In addition, we prepared W/O/W insulin emulsions containing Pluronic F 127 (PF 127) gel in order to control the duration of hypoglycemic effects of the emulsion, and evaluated the effects on the profiles of hypoglycemia. Our previous study (Suzuki et al., 1998) showed that the emulsion incorporating DHA did not induce any damage in the structure of the intestinal mucosa in a single dose administration. However, the insulin formulations are used chronically in practice. Therefore, we carried out a morphological evaluation of the intestinal damage caused by multiple application of these emulsions.

2. Materials and methods

2.1. Materials

Crystalline porcine insulin (27.3 IU/mg) was kindly supplied by Simizu Pharmaceutical (Shizuoka, Japan). Gelatin, a glucose B-Test kit, triolein and sorbitan monooleate (Span 80), DL-α-tocopherol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Polyoxyethylene sorbitan monooleate (Tween 80) and oleic acid (purity: 99.0%) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Egg yolk phospholipids (phosphatidylcholine and phosphatidylethanolamine) were purchased from Nippon oil & Fats (Tokyo, Japan). Stearic acid (purity: 99.0%) and Pluronic F 127 were purchased from Sigma (St Louis, MO). Docosahexaenoic acid (purity: 99.0%) and eicosapentaenoic acid (purity: 99.0%) were provided by Nippon Suisan Kaisya (Tokyo, Japan). All other chemicals were of analytical grade and commercially available.

2.2. Preparation of W/O/W emulsion

W/O/W emulsions were prepared by a two-step emulsification procedure using a homogenizer (Ace Homogeniser, Nihonseiki Kaisha Tokyo, Japan) according to the method reported in a

previous paper (Morishita et al., 1998). Briefly, weighed amounts of insulin were dissolved in 200 μl of 0.1 N HCl, and then phosphate-buffered saline (PBS, pH 7.4) containing gelatin (5% of the inner aqueous phase) was added to the solution. The pH value of the solutions was adjusted to pH 7.4 by the addition of 0.1 N NaOH as required. The weight of aqueous phase was finally adjusted to 2 g. The oily phase (8 g) was composed of 0.06% DL-α-tocopherol, 5% egg yolk phospholipids (phosphatidylcholine: phosphatidylethanolamine = 7:3), 2.5, 5, 10 or 20% free fatty acids, 20% Span 80, and 54.94–72.44% triolein. Thirty grams of purified water containing 3% Tween 80 was used for the outer aqueous phase. Thus, the weight ratio of each phase was as follows: inner aqueous phase:oily phase:outer aqueous phase = 1:4:15.

2.3. Preparation of W/O/W emulsion containing PF 127

A stock PF 127 solution (30%) was prepared firstly by the cold method (Barichello et al., 1999). A weighed amount of PF 127 was slowly added to an aqueous (5–10°C) solution with gentle mixing until complete dissolution of the polymer. W/O/W insulin emulsions were prepared as described above, and then added to the PF 127 solution in the weight ratio of 1 to 1. The final concentration of PF 127 was 15% of the formulation weight.

2.4. In vivo single and multiple absorption experiments

Male Wistar rats weighing 180–220 g were fasted for 48 h prior to the experiments. During the experimental period, rats were housed in cage and were given water ad libitum except for the sampling time. Rats were restrained in a supine position during administration and at each blood sampling under non-anesthesia. The emulsion was administered rectally through a stomach sonde needle for rats (KN-348, Natsume Seisakusyo Ltd., Tokyo, Japan). In the single absorption study, two insulin doses, 5 and 10 IU/kg body weight (the weights of the emulsion were 0.5 and 1.0 g/kg, respectively) were used. The rats restrained in a supine position on a board and a 0.2-ml sample of blood was taken from the jugular vein 5 min before administration. Subsequent blood samples were taken at 15, 30, 60, 120, 240 and 360 min after dosing. Serum was separated by centrifugation at 13 000 rpm for 1 min and kept frozen until analysis.

In multiple administration experiments, the emulsion was administered once a day at 10:00 h for 10 days. The dose of insulin was fixed at 5 IU/kg body weight. On the first and the tenth day of the experiment, blood samples were taken as described above.

The serum glucose level was determined by using a glucose B-Test kit. Post-dose levels of the serum glucose were expressed as a percentage of the pre-dose level. The percentage change in the serum glucose levels was taken as the percentage of the pre-dose level subtracted from 100. The cumulative percentage change in the serum glucose level was calculated by summing, using the trapezoidal method, the areas below baseline levels in the percentage change versus time curves for 0–4 h ($AUC_{glucose}$). The serum insulin levels were determined using an enzyme immunoassay (IMX System, DAINABOT, Tokyo, Japan). The basal endogenous insulin level was subtracted from all insulin levels measured following insulin administration. The area under the insulin level-time curves for 0–4 h ($AUC_{insulin}$) was determined using the trapezoidal rule.

The efficacy of the enterally administered insulin was calculated relative to that administered by the s.c. route using methods described by Morishita et al. (1998). Briefly, insulin solutions were prepared by dissolving an appropriate amount of crystalline porcine insulin in PBS. The s.c. insulin doses were 0.5, 1.0, 2.0 and 3.0 IU/kg body weight. Blood samples were collected from the jugular vein before and at 5, 15, 30, 60, 120, 240 and 360 min after dosing. The relationship between the logarithm of s.c. insulin doses and $AUC_{glucose}$ was obtained from the results of the s.c. administration study. The pharmacological availabilities were calculated by using the dose/response curve.

The experimental procedures described above were performed according to the rules set by the

Committee on Ethics in the Care and Use of Laboratory Animals in Hoshi University.

2.5. In vivo absorption experiments using W/O/W emulsion containing PF 127

Male Wistar rats weighing 180–220 g were fasted for 48 h prior to the experiments and were anesthetized by an i.p. injection of 50 mg/kg sodium pentobarbital. The rats were restrained in a supine position on a board which was kept at a surface temperature of 37°C. In this study, the dose of insulin was 20 IU/kg body weight. Administration of insulin emulsion and the sampling of blood were done as described above.

2.6. Microscopic study

The rectum was excised after 10 days administration of various emulsions. The isolated intestine was fixed in 10% neutral carbonate-buffered formalin, paraffin-sectioned, and stained with hematoxylin and eosin. All tissues were analyzed by light microscopy and scored based on histopathological findings.

2.7. Statistical analysis

Each value was expressed as the mean $\pm$ S.D. For group comparisons, the one-way layout ANOVA with duplication was applied. Significant differences in the mean values were evaluated by Student's unpaired t-test. A P value of >0.05 was considered significant.

3. Results

3.1. Changes in serum insulin and glucose levels after administration of insulin emulsion incorporating unsaturated fatty acid

Fig. 1 shows the changes in serum glucose and insulin levels following the in vivo administration of 5 and 10 IU/kg dose of insulin emulsions incorporating various fatty acids (2% of the total emulsion) into the rectum. A stearic acid, C18 saturated fatty acid, was used as a control in this study. As shown in Fig. 1, insulin absorption from the rectum was enhanced markedly, and a marked hypoglycemic effect was induced by unsaturated fatty acid such as oleic acid, EPA and DHA. The peak insulin levels and lowest glucose levels appeared showed at 0.25–0.5 and 0.5 h after administration, respectively. Fig. 2 shows the relationship between insulin dose and the mean $AUC_{glucose}$ following administration of emulsions. It clearly shows that insulin emulsion had dose-related hypoglycemic effects. Serum insulin levels increased and glucose levels decreased in an insulin dose-related fashion. Notably, the emulsions incorporating EPA or DHA showed a significant relationship between insulin dose and $AUC_{glucose}$. The mean $AUC_{glucose}$ and pharmacological availabilities of various emulsions are given in Table 1. The pharmacological availability of the emulsion incorporating DHA was $\approx 25\%$, and it was two or three times greater than the emulsion incorporating EPA or oleic acid, respectively.

3.2. Effects of DHA amount in the emulsion on insulin absorption enhancement

We evaluated the effect of various concentrations of DHA in the emulsions on the insulin absorption. Fig. 3 shows the mean $AUC_{insulin}$ and $AUC_{glucose}$ following the administration of 5 IU/kg of insulin emulsion incorporating various amounts of DHA. At 0.5% DHA, $AUC_{insulin}$ and $AUC_{glucose}$ values were reduced to $\approx 30\%$ ($P < 0.05$) and 60% of those at 1% DHA, respectively (Fig. 3). In the range beyond 1%, no further enhancement of the insulin absorption proportional to the amount of DHA was observed.

3.3. Effect of PF 127 on the hypoglycemic profiles of insulin emulsion

Fig. 4 shows the effect of PF 127 on serum glucose level after administration of insulin emulsion incorporating 2% DHA into rat rectal under anesthesia. In insulin emulsion, the serum glucose level decreased rapidly but had recovered to the base-line after 4 h of administration. The mean maximum decreases of blood glucose at 5 and 20 IU/kg doses of insulin were 75.9 and 61.1% of the

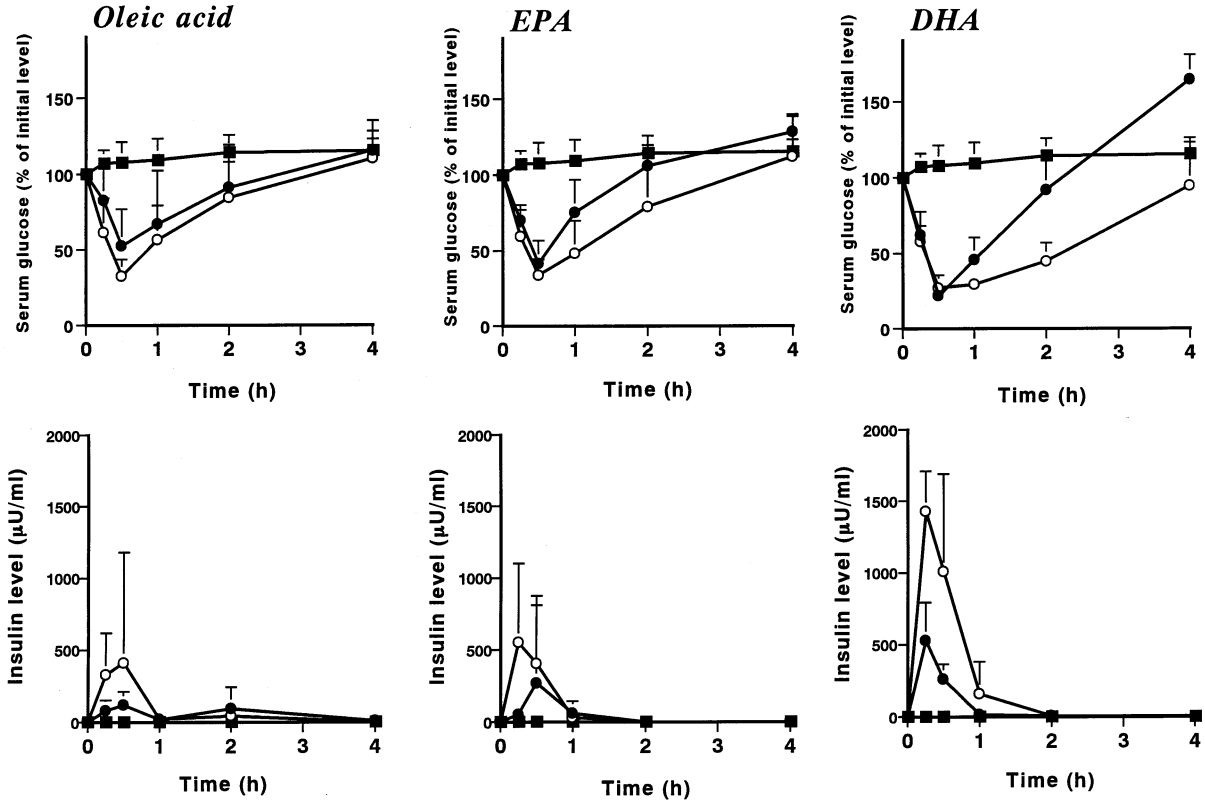

Fig. 1. Effect of rectal administration of insulin emulsion incorporating 2% unsaturated fatty acid on serum glucose and insulin levels. Serum glucose and insulin levels after application of the insulin emulsion in insulin doses of 5 IU/kg (●) and 10 IU/kg (○) for EPA and DHA, 5 IU/kg (■) for stearic acid.

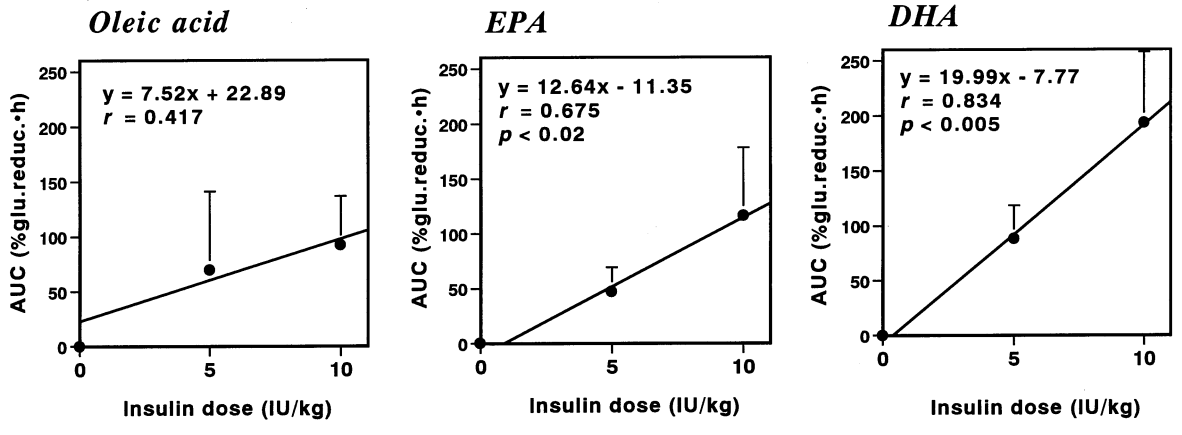

Fig. 2. Relationship between insulin dose and $AUC_{glucose}$.

Table 1
Pharmacological availability of insulin emulsion incorporating unsaturated fatty acid[a]

	AUC (% glu. reduc. × h)	*F* (%)
Oleic acid	92.3 ± 44.4	7.7 ± 3.3
EPA	116.6 ± 61.6	11.0 ± 7.0
DHA	193.7 ± 63.8	25.4 ± 17.0

[a] Insulin dose: 10 IU/kg. Each value represents the mean ± S.D.

initial level, and the mean T_{max} values were 1.2 and 1.0 h, respectively. In contrast, insulin emulsion containing PF 127 induced a gradual decrease in the serum glucose level, and this effect was maintained up to 6 h after administration. The nadir of the serum glucose-time curve was broadened. The mean T_{max} value was 1.8 and 2.0 h at 5 and 20 IU/kg of insulin, respectively. These profiles were obtained for both high and low doses of insulin administration, but were more obvious at high insulin dose.

3.4. Hypoglycemic effect of multiple administrations of insulin emulsion incorporating oleic acid or DHA

Fig. 5 shows the hypoglycemic effect of multiple administrations of insulin emulsion incorporating oleic acid or DHA. Insulin emulsion without unsaturated fatty acids was used as a control in this study. The hypoglycemic effects of insulin emulsion incorporating oleic acid or DHA were evaluated on the first and the tenth day of the multiple dose study. For the emulsion incorporating DHA, the mean $AUC_{glucose}$ values on the first and the tenth day were almost the same, 45.7 and 54.8% AUC (% glu. reduc. × h), respectively. Although AUC values did not change during multiple administration, the nadir of the glucose level-time curve tended to increase. Similar trends were observed for the emulsion incorporating oleic acid.

3.5. Morphological appearance of the mucosal surface

The morphological appearance of the rectal mucosa after multiple applications of the emulsion incorporating oleic acid and DHA was analyzed by light microscopy (Fig. 6). Tissue damage scores based on histopathological findings are summarized in Table 2. Control and insulin emulsion incorporating oleic acid did not produce any mucosal damage in the rectum. Similarly, insulin emulsion incorporating DHA induced no or very little mucosal damage.

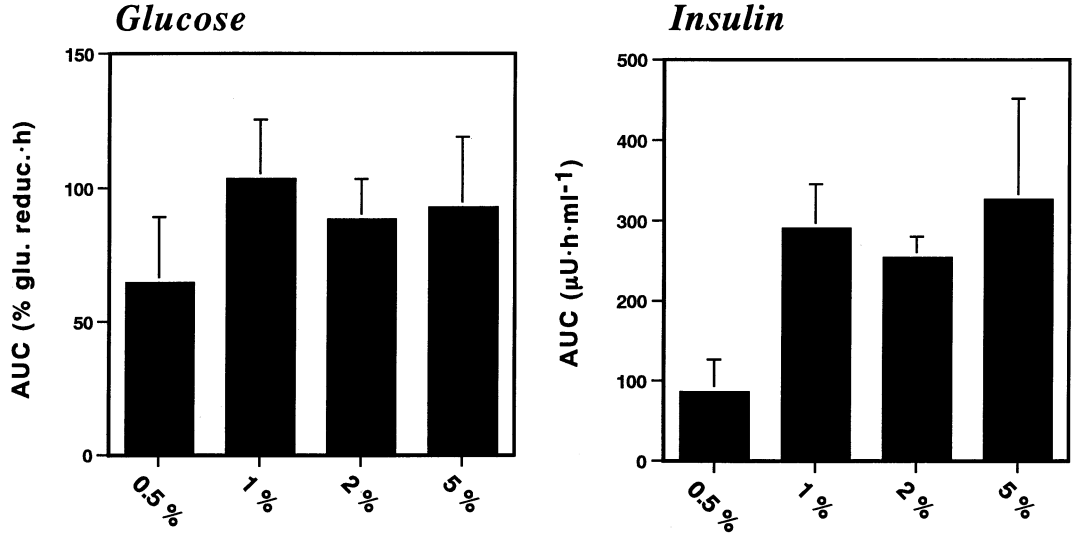

Fig. 3. Comparison of $AUC_{glucose}$ and $AUC_{insulin}$ obtained from insulin emulsion incorporating various concentrations of DHA.

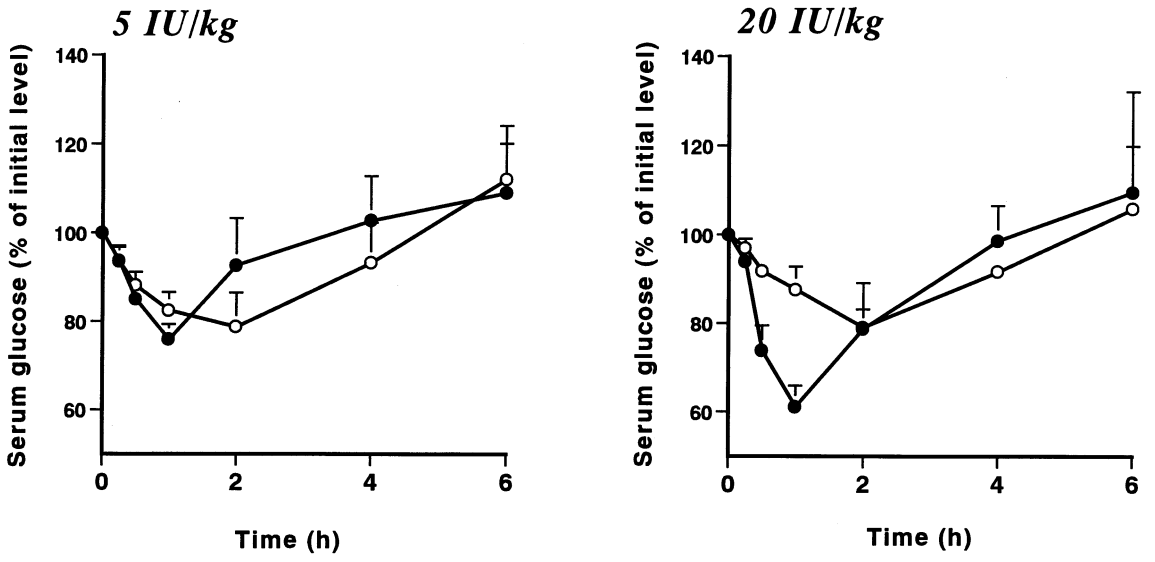

Fig. 4. Effect of Pluronic F127 on serum glucose levels after administration of insulin emulsion incorporating DHA. Serum glucose level after application of the insulin emulsion with (○) or without (●) Pluronic F 127.

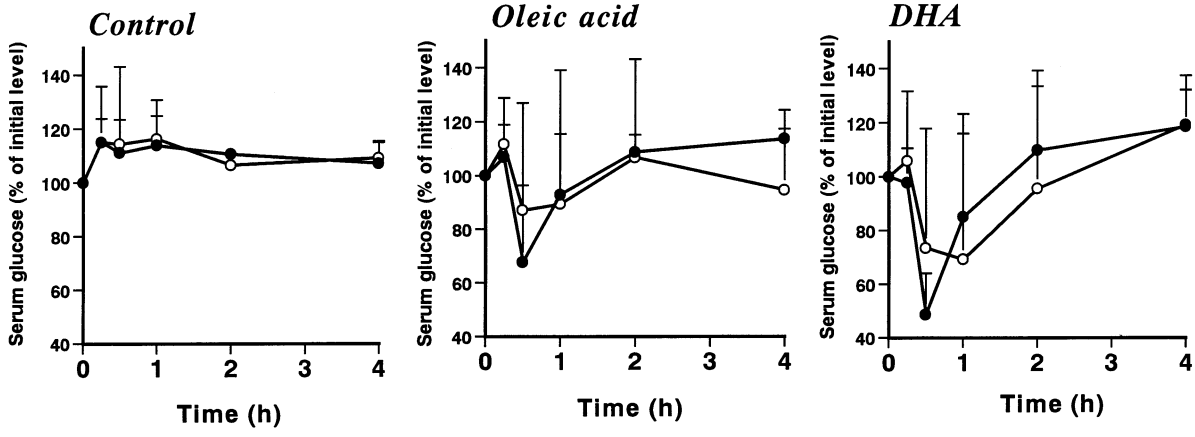

Fig. 5. Hypoglycemic effect of multiple administrations of insulin emulsion incorporating various fatty acids. Serum glucose level after application of the insulin emulsion on the first day (●) and the tenth day (○) at an insulin dose of 5 IU/kg.

4. Discussion

Our previous study using the in situ loop method has demonstrated that W/O/W emulsions incorporating unsaturated fatty acid such as oleic acid (Morishita et al., 1998; Suzuki et al., 1998), EPA and DHA (Suzuki et al., 1998) promote the absorption of insulin from rats intestine. In the present study, we carried out insulin absorption experiments to confirm the enhancing effect of highly purified polyunsaturated fatty acid in vivo. In the rectum, these emulsions enhanced insulin absorption and induced marked hypoglycemic effects (Fig. 1). Particularly, the enhancing effect of DHA was strong among fatty acids used in the study. These results coincided with our previous study (Morishita et al., 1998; Suzuki et al., 1998). Moreover, our results indicated that the hypo-

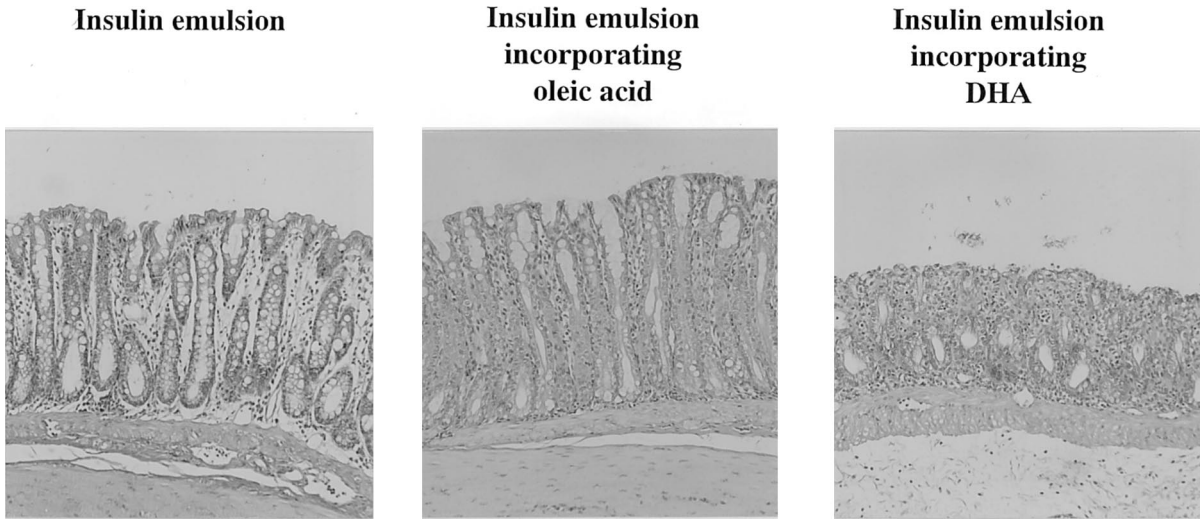

Fig. 6. Light micrographs (H & E stain, × 100) of rat rectal mucosa following 10 days multiple application of various emulsions.

glycemic effect induced by unsaturated fatty acids was insulin dose-dependent (Fig. 2).

Further, we compared the enhancing effects of various DHA concentrations incorporated into insulin emulsion in this study. Our previous study showed that C18 unsaturated fatty acids did not promote the absorption of insulin from intestine at below 2% in the emulsion (Morishita et al., 1998). By contrast, DHA strongly promoted insulin absorption even at 1% in the emulsion. $AUC_{insulin}$ and $AUC_{glucose}$ values of that emulsion were equal to those of the emulsion incorporating 2% DHA (Fig. 3). Therefore, these results suggested that the insulin absorption enhancing effect of DHA occurred at smaller amounts than that of C18 unsaturated fatty acids. It is thought that the absorption enhancement effect depends on the concentration of absorption enhancers (Muranishi, 1990). However, $AUC_{glucose}$ values did not change when the amount of DHA incorporated increased to 5%. It was recently suggested that the intestinal uptake of long-chain unsaturated fatty acids might be carrier mediated (Gore et al., 1994; Prieto et al., 1996). Moreover, saturation in the capacity for absorption of EPA or DHA was suggested (Hawthorne et al., 1990; Krokan et al., 1993). Thus, it is likely that a certain amount of fatty acid is required to enhance insulin absorption, however there might be a limit for uptake into the membrane.

Some unsaturated fatty acids, such as oleic acid, are abundant in nature, and alter the permeability of intestinal epithelial cells (Velasquez et al., 1993). It has been reported that the enhancement effect induced by oleic acid accompanies mucosal irritation (Velasquez et al., 1993; Ishikawa et al., 1994). On the other hand, we have reported that DHA did not induce any mucosal damage on single application (Suzuki et al., 1998).

Table 2
Microscopical findings of the rectum after multiple doses of insulin emulsions incorporating fatty acid[a]

Microscopic findings	Fatty acid		
	None	Oleic acid	DHA
Mucosa			
Degeneration	0	0	1
Lamina propria mucosa			
Cellular infiltration	0	0	1
Fibrosis	0	0	0
Submucosa			
Edema	0	0	0
Cellular infiltration	0	0	0

[a] Score: 0, no change; 1, very slight; 2, slight; 3, moderate; 4, marked.

Similar results were reported by Horie et al. (1998). DHA protected the small intestine from the mucosal damage caused by oral administration of the antitumour drug, methotrexate. In this multiple administration study, mucosal damage by the emulsion incorporating unsaturated fatty acid was also not or hardly seen (Fig. 6 and Table 2). Thus, DHA is thought to be an attractive candidate as an absorption enhancer for the enteral route because of its strong permeability enhancement and less toxicity.

The time course of the glucose reduction provided by the insulin emulsion incorporating polyunsaturated fatty acids was very rapid, and the profiles are similar to that of the only insulin monomeric analogue, insulin lispro (Howey et al., 1994). It was reported that the pharmacokinetic and pharmacodynamic profiles of insulin lispro mimic closely those of physiological mealtime insulin (Hoffman and Ziv, 1997). In addition to the rapid-acting formulation, long-acting formulations are required in order to generate basal insulin levels. Gel formulation has advantages for sustained drug release because it of its adhesiveness and prolongation effect of the contact time to the mucosal membrane. Kimura et al. (1996) reported that gel spheres containing insulin prolonged residence time in the small intestine. To give the emulsion an adhesive property, we prepared insulin emulsions containing amphiphilic block copolymer, PF 127, and carried out insulin absorption experiments in vivo. Pluronic block copolymers are nonionic surfactants and exhibit reversible thermal gelation at concentrations above the CMC (Barichello et al., 1999). Fig. 4 clearly shows that a prolonged hypoglycemic effect was induced by insulin emulsion containing PF 127. These results suggested that it is possible to design a variety of formulations, rapid and long-acting preparations, using the emulsion and PF 127.

In conclusion, rectal administration of insulin emulsion incorporating unsaturated fatty acids, particularly DHA, induced a marked hypoglycemic effect and insulin absorption from the rectum occurred in an insulin dose-related fashion. Moreover, the hypoglycemic effect induced by insulin emulsion could be sustained by adding PF 127 to the emulsion. In the multiple administration study, stable profiles of hypoglycemia were obtained without gross mucosal damage throughout the experimental period.

Acknowledgements

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