Enhanced Bioavailability of Subcutaneously Injected Insulin by Pretreatment with Ointment Containing Protease Inhibitors[1]

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The present study was undertaken to develop an ointment preparation containing a protease inhibitor for stabilizing subcutaneously injected insulin. The ointment containing the protease inhibitor, gabexate mesilate or nafamostat mesilate, was applied to the skin around the insulin injection site. Three results were obtained. First, gabexate and nafamostat inhibited insulin degradation in subcutaneous tissue homogenates *in vitro*. Second, after application of gabexate or nafamostat ointment, an appreciable amount of gabexate or nafamostat appeared in the subcutaneous tissue of rats or hairless mice and their concentrations were comparable to those seen in the *in vitro* experiment. Third, insulin degradation at the subcutaneous injection site in the rat was depressed after pretreatment with gabexate or nafamostat ointment. Pretreatment with gabexate or nafamostat ointment increased the plasma immunoreactive insulin (IRI) levels and the hypoglycemic effect of insulin in healthy volunteers. These results indicate that gabexate or nafamostat ointments stabilize subcutaneously injected insulin.

KEY WORDS: insulin; subcutaneous administration; protease inhibitor; gabexate; nafamostat; ointment; bioavailability.

INTRODUCTION

Recently, many biologically active peptides have been discovered and have attracted attention as new drugs (1). Clinical dosage forms of these peptides have been primarily parenteral forms due to transport and enzymatic barriers (2). Although subcutaneous injection is often employed in peptide therapeutics, these peptides are susceptible to degradation by proteolytic enzymes at the injection site (3–12).

We have previously reported the absorption and degradation of porcine and human insulin at subcutaneous injection sites in rats and humans (13–16). In these studies, we demonstrated that certain protease inhibitors suppress degradation of subcutaneously injected insulin. Substrates of collagenase-like peptidase (benzyloxycarbonyl-Gly-Pro-Leu-Gly) (13) and atelo collagen amplify the bioavailability of subcutaneously injected insulin.

Guanidinobenzoate derivatives have inhibitory effects on various proteolytic enzymes (17–21). These protease inhibitors are used clinically in the treatment of acute pancreatitis and shock (22). Gabexate ointment is used in the treatment of traumatic inflammation. The stabilizing effects of an ointment containing gabexate mesilate [ethyl-4-(6-guanidino hexanoyloxy) benzoate methane sulfonate] and the structurally related nafamostat mesilate (6-amidino-2-naphthyl *p*-guadininobenzoate dimethanesulfonate) were tested in this study.

MATERIALS AND METHODS

Materials and Preparation of Ointment

Monocomponent porcine insulin (26.0 U/mg, Novo Industri A/S, Copenhagen, Denmark) and Actrapid and Semilente insulin (40 U/ml) were used. Gabexate mesilate [ethyl-4-(6-guanidino hexanoyloxy) benzoate methane sulfonate] and nafamostat mesilate (6-amidino-2-naphthyl *p*-guadininobenzoate dimethanesulfonate; Futhan) were kindly supplied by Ono Pharmaceutical Co. (Osaka, Japan) and Torii Pharmaceutical Co. (Osaka, Japan), respectively. Futhan Injection (containing lyophilized nafamostat mesilate) was also used. ^{125}I-Insulin was prepared by a modification of the chloramine-T method (23). The labeled insulin was purified twice by gel filtration using Sepadex G-25 and G-50. The specific activity of insulin was 120 mCi/mg. For the preparation of gabexate (1%, w/w) or nafamostat (0.5%, w/w) ointment, gabexate or Futhan Injection was triturated, then liquid paraffin and Azunol ointment (lanolin:white vaseline = 50:50 w/w, Nippon-Shinyaku, Kyoto, Japan) were added and mixed.

Animals and Subjects

Male Wistar rats weighing 120–180 g and hairless mice weighing 20–30 g were used. During the experiments, rats and hairless mice were anesthetized with pentobarbital (40 mg/kg, *ip*). The rectal temperature was monitored and maintained at a constant (36.5 ± 0.5°C) by keeping the animals in a temperature-constant box (13). Diabetes was induced in the rats by intravenously injected 50 mg/kg streptozotocin (Sigma, St Louis, MO) (24). Healthy volunteers of normal body weight, aged 22–44, were used in this study. Consent was obtained from all volunteers, who were fully informed of the experimental nature of the study. The study was approved by the Kyoto University Hospital Ethical Committee.

Assay of Insulin Degrading Activity *in vitro*

Dorsal skin was removed from anesthetized rats, and then the stratum corneum was carefully removed using a surgical knife. The subcutaneous tissue which included epi-

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dermis, dermis, and adipose tissue was homogenized in 0.25 *M* sucrose in a glass homogenizer cooled by ice-cold water. The insulin degrading activity was estimated by a modification of the method of Hammond *et al.* (25) on the basis of trichloroacetic acid (TCA)-precipitable radioactivity produced by incubation with labeled insulin. The incubation mixture (0.55 ml) contained porcine insulin (1×10^{-8} *M*), a tracer level of ^{125}I-insulin, subcutaneous tissue homogenate (50 μg of protein), and 0.5% bovine serum albumin in Krebs–Ringer bicarbonate buffer (pH 7.4) with or without a protease inhibitor. After incubation at 37°C for 15 min, the reaction mixture was precipitated by the addition of 10% TCA and centrifuged at 4°C. Radioactivity in the supernatant and precipitate was counted in a gamma-counter. The percentage of insulin degraded was calculated from the increase in radioactivity in the supernatant compared to control tubes incubated without subcutaneous tissue. The protein concentration was measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Analysis of Gabexate and Nafamostat Concentrations in Subcutaneous Tissue of Rats and Hairless Mice

The depilated dorsal or abdominal skin (4-cm^2 area) was treated with gabexate (1%) or nafamostat (0.5%) ointment. At 1, 2, and 3 hr after the application of gabexate or nafamostat ointment, the skin of the treated area was excised, the skin was isolated, and the subcutaneous tissue without the stratum corneum was prepared. Gabexate concentration in the subcutaneous tissue was determined by HPLC (C_{18} reverse-phase μ-Bondapak column) (26). Sodium acetate (pH 3.8)–acetonitrile (50:50, v/v) was used as a mobile phase at a flow rate of 1.5 ml/min. Absorbance was monitored at 237 nm. The nafamostat concentration in the subcutaneous tissue was also determined by HPLC (27).

Measurement of Insulin Degradation at the Injection Site in Cardiac-Arrested Rats

The degradation in cardiac-arrested rats was investigated to estimate the degradation rate of insulin at the injection site when its absorption would be negligible. This condition was induced by direct injection of pentobarbital to the heart 5 sec before the experiment (13). No absorption of insulin from the subcutaneous injection site was assumed to occur in this condition because the systemic circulation was stopped completely.

Two symmetrical areas (4 cm^2) of the depilated abdominal skin were demarcated. One side was treated with gabexate or nafamostat ointment; the other side, with ointment vehicle (Azunol ointment). At 1 hr after the application of the ointment, intracardiac injection of pentobarbital (200 mg/kg) was used to induce cardiac arrest (13). Ten microliters of insulin solution (0.2 U/kg) containing ^{125}I-insulin were injected into each subcutaneous site immediately after intracardiac injection of pentobarbital. At 30 min after insulin injection, each skin sample (4 cm^2) and the associated subcutaneous tissue were homogenized in 10% TCA and centrifuged. The TCA-soluble and -precipitable radioactivities were determined. In a separate control group, the skin sample and subcutaneous tissue were excised immediately after insulin injection, and the percentage of degraded insulin was calculated as noted above. Recoveries of radioactivity were more than 95%.

Determination of Bioavailability of Subcutaneously Injected Insulin in Rats

One gram of gabexate ointment or ointment vehicle was applied to the depilated dorsal skin (4 cm^2) in 16-hr fasted rats. At 30 min after the application of the ointment, Actrapid insulin (0.2 U/kg, 10 μl) was injected into the treated skin and plasma IRI and glucose levels were monitored for 40 min. One gram of nafamostat (0.5%) ointment or ointment vehicle was applied to the abdominal skin (4 cm^2) in diabetic rats fasted for 16 hr. At 1 hr after the application, Semilente insulin (0.4 U/kg, 10 μl) was injected into the ointment-treated site. Plasma IRI and glucose levels were monitored for 3 hr after the administration. Plasma IRI was estimated using an enzyme immunoassay kit (Wako-Pure Chemicals, Osaka). Plasma IRI levels measured in a control experiment, which involved subcutaneous bolus saline injection into the rats, were less than 2 μU/ml, indicating that this enzyme immunoassay system features poor cross-reactivity to rat insulin. The plasma glucose was measured by the *o*-toluidine method (28).

Determination of Bioavailability of Subcutaneously Injected Insulin in Healthy Volunteers

Nine healthy volunteers (within 10% of ideal body weight; 23–44 years of age; four males and five females) were fasted from 22:00 on the day before the experiment to the end of the experiment. Two grams of gabexate (1%) or nafamostat (0.1%) ointment was applied to the upper arm (9 cm^2) at 8:00 in the morning. For crossover experiments, controls were applied with the ointment vehicle only. At 30 min after the application, Actrapid insulin (0.1 U/kg) was administered subcutaneously. Plasma IRI and glucose levels were monitored for 3–5 hr after the administration.

Statistical Analysis

Statistical analysis was performed using Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Degradation of Insulin in Subcutaneous Tissue Homogenates and at the Subcutaneous Injection Site in the Cardiac-Arrested Rats

The inhibitory effect of nafamostat mesilate on *in vitro* ^{125}I-insulin degradation was compared with that of gabexate mesilate. As shown in Fig. 1, gabexate mesilate and nafamostat mesilate inhibited ^{125}I-insulin degradation in rat subcutaneous tissue homogenates in a dose-dependent manner. Nafamostat mesilate was more effective than gabexate in decreasing TCA-soluble radioactivity generation.

The percentage change in TCA-precipitable radioactivity at the injection site was examined in cardiac-arrested rats at 30 min after subcutaneous injection of ^{125}I-insulin. This confirmed that degradation of ^{125}I-insulin at the subcutaneous injection site occurs *in vivo*. Pretreatment with nafamostat ointment decreased the percentage of TCA-soluble radioactivity; 12.3 ± 2.3% of the total radioactivity in the skin

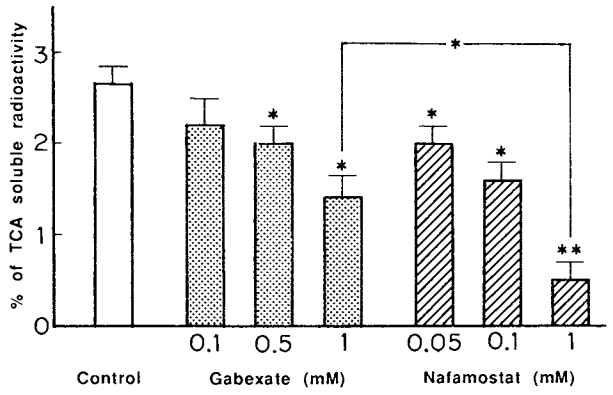

Fig. 1. Effect of gabexate and nafamostat on generation of TCA-soluble radioactivity after ^{125}I-insulin was incubated with a rat subcutaneous tissue homogenate for 15 min at 37°C with or without protease inhibitor. Degradation was determined as described under Materials and Methods. Controls were incubated without subcutaneous tissue. Results are shown as mean ± SE of three experiments. Statistical significance: (*) $P < 0.05$ and (* *) $P < 0.01$ compared to controls.

treated with ointment vehicle only was TCA soluble, whereas only 6.6 ± 2.4% of the total radioactivity was TCA soluble in the skin treated with nafamostat ointment ($n = 7$).

Concentrations of Gabexate or Nafamostat in the Subcutaneous Tissue After Topical Application of Gabexate or Nafamostat Ointment

After topical application of gabexate ointment, an almost-constant concentration of gabexate was observed in the subcutaneous tissue of rats and hairless mice over 1 to 3 hr (Table I). One hour after the application of nafamostat ointment, 3–20 μg/g tissue of nafamostat was detected in the subcutaneous tissue of rats and hairless mice (Table II). Both rats and hairless mice had noticeably higher concentrations of nafamostat in the abdominal tissue than in the back tissue. The concentration of nafamostat in the subcutaneous tissue of the hairless mice was two times higher than in the rats. Three hours after application of the nafamostat ointment in the rat, 2.44 ± 0.40 and 7.13 ± 1.20 μg/g tissue of nafamostat were detected in the back and abdominal tissue, respectively.

Effect of Topical Application of Gabexate and Nafamostat Ointment on the Bioavailability of Subcutaneously Injected Insulin in Rats and Humans

When insulin (0.2 U/kg) was injected subcutaneously into rat skin treated with gabexate ointment, the plasma IRI levels showed a tendency to exceed those observed in rats treated with the ointment vehicle. The hypoglycemic effect of insulin in the gabexate-treated rats was also greater than that of the controls (Fig. 2). When Semilente insulin (0.4 U/kg) was injected subcutaneously into the diabetic rat treated with nafamostat ointment, the plasma IRI levels showed a tendency to exceed those observed in diabetic rats treated with the ointment vehicle only. The hypoglycemic effect of insulin in these animals was not significantly different than the controls (Fig. 3).

To confirm these preclinical results, a preliminary study in healthy volunteers was conducted. When Actrapid insulin (0.1 U/kg) was administered subcutaneously to four volunteers at a site previously treated with gabexate ointment, plasma IRI levels were higher than those of the controls, but the hypoglycemic effect of insulin was not significantly different from the controls in the crossover experiment (Fig. 4). When Actrapid insulin (0.1 U/kg) was administered subcutaneously in five healthy volunteers at a site previously treated with nafamostat ointment, the plasma IRI levels were higher than those of the controls in the crossover experiment, and the hypoglycemic effect of insulin was prolonged (Fig. 5).

Table I. Concentration of Gabexate in the Subcutaneous Tissue of the Rat[a]

After application (hr)	Concentration (μg/g tissue)
1	4.57 ± 0.42
2	3.45 ± 0.59
3	3.53 ± 1.19

[a] The values represent the mean ± SE of three to five animals in each group.

Table II. Concentration of Nafamostat in the Subcutaneous Tissue of Rats and Hairless Mice at 1 hr After Application[a]

		Concentration (μg/g tissue)	
	N	Back	Abdominal
Rat	5	3.26 ± 0.82	8.33 ± 2.83
Hairless mouse	3	6.29 ± 0.75	19.7 ± 4.72

[a] The values represent the mean ± SE.

DISCUSSION

This study demonstrated that the protease inhibitors gabexate or nafamostat mesilate can decrease the generation of TCA-soluble radioactivity after subcutaneous injection of ^{125}I-insulin, suggesting that insulin degradation is decreased.

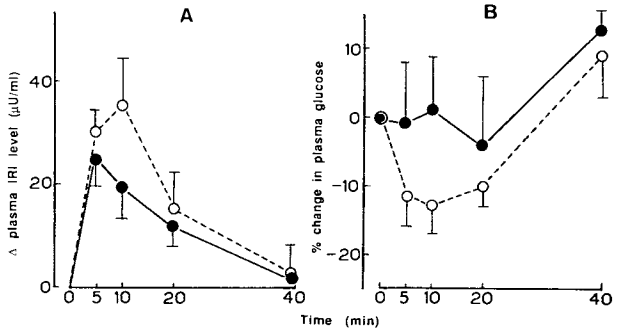

Fig. 2. Effect of gabexate ointment on plasma IRI levels and hypoglycemic effect after injecting insulin subcutaneously in rats. Insulin (0.2 U/kg) was injected into the skin treated with gabexate or vehicle ointment. (A) Plasma IRI levels; (B) percentage change in plasma glucose levels. Each point represents the mean value of five experiments. Vertical bars indicate SE. (●) Insulin + ointment vehicle; (○) insulin + gabexate ointment.

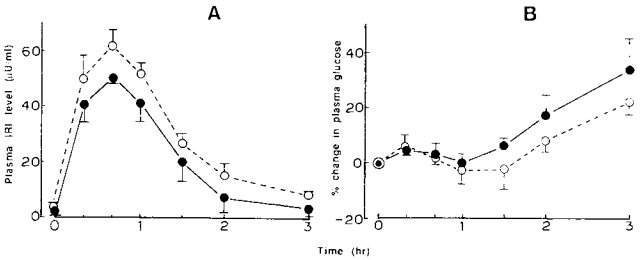

Fig. 3. Effect of nafamostat ointment on plasma IRI levels and hypoglycemic effect after injecting insulin subcutaneously in diabetic rats. Semilente (0.4 U/kg) was injected into the skin treated with nafamostat or vehicle ointment. (A) Plasma IRI levels; (B) percentage change in plasma glucose levels. Each point represents the mean value of three or four experiments. Vertical bars indicate SE. (●) Insulin + ointment vehicle; (○) insulin + nafamostat ointment.

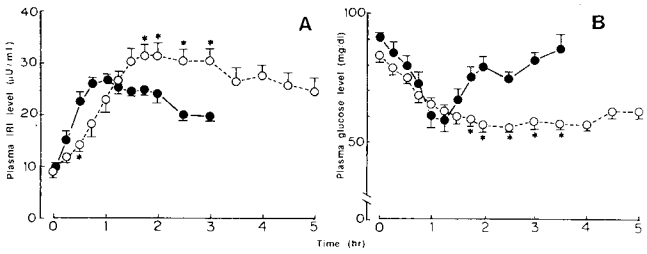

Fig. 5. Effect of nafamostat ointment on plasma IRI levels and hypoglycemic effect after injecting insulin subcutaneously in five healthy volunteers. Actrapid (0.1 U/kg) was injected into the skin treated with nafamostat or vehicle ointment. (A) Plasma IRI levels; (B) plasma glucose levels. Vertical bars indicate SE. (●) Insulin + ointment vehicle; (○) insulin + nafamostat ointment. Statistical significance: (*) $P < 0.05$.

These protease inhibitors have strong inhibitory effects on a variety of proteases, such as trypsin, plasmin, and kallilrein (21). Nafamostat, the more potent protease inhibitor, was more effective than gabexate in the apparent suppression of insulin degradation *in vitro*. The degradation of insulin at the subcutaneous injection site in the cardiac arrested rats was approximately four times greater than its degradation *in vitro*.

Formulating gabexate and nafamostat in an ointment has several advantages. First, gabexate and nafamostat contained in an ointment are released continuously and depress proteolytic enzyme activity in the subcutaneous tissue for a long time. Our data suggest that gabexate and nafamostat can permeate skin easily. The satisfactory permeability of gabexate and nafamostat may be owing to their low molecular weight (approximately 400). Second, the application of an ointment is generally easy and convenient. In addition, gabexate is stable for more than 1 month in an oleaginous ointment vehicle such as that used in this study (29,30).

Nafamostat and gabexate increased plasma IRI levels and nafamostat prolonged the hypoglycemic effect of subcutaneously injected insulin in normal volunteers when it was applied to the skin at the insulin injection site in the form of an ointment. Similar results were observed in the rat, except that gabexate appeared to have the more dramatic effect on the response. These results may indicate that the skin permeations of these protease inhibitors are different between

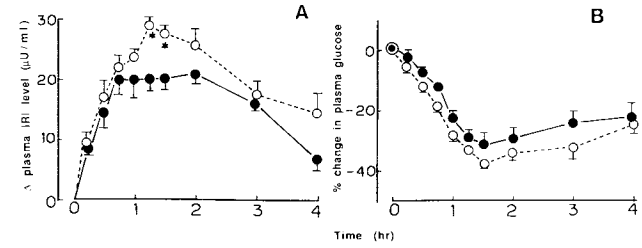

Fig. 4. Effect of gabexate ointment on plasma IRI levels and hypoglycemic effect after injecting insulin subcutaneously in four healthy volunteers. Actrapid (0.1 U/kg) was injected into the skin treated with gabexate or vehicle ointment. (A) Plasma IRI levels; (B) percentage change in plasma glucose levels. Vertical bars indicate SE. (●) Insulin + ointment vehicle; (○) insulin + gabexate ointment. Statistical significance: (*) $P < 0.05$.

rat skin and human skin. Previous work (31–34) indicates a similarity in the barrier properties of hairless mouse and human skins. Higher subcutaneous nafamostat concentrations were achieved in the hairless mouse than in the rat after the application of nafamostat ointment. A difference in penetration may explain the difference observed between rats and humans in stabilization of subcutaneous insulin by nafamostat.

The absorption of insulin in normal volunteers was delayed when nafamostat ointment was applied to the injection site of human skin (Fig. 5). Possible explanations include the formation of a nafamostat–insulin complex and/or a decrease in subcutaneous blood flow caused by the nafamostat ointment. These possibilities are being investigated.

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