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Promoting effect of the new chymotrypsin inhibitor FK-448 on the intestinal absorption of insulin in rats and dogs

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FK-448 is a potent and specific inhibitor of chymotrypsin, which enhances the intestinal absorption of insulin in rats and dogs resulting in a decrease in blood glucose levels in these animals. In dogs, the immunoreactive insulin (IRI) level of plasma rose proportionally to the decrease in blood glucose level. From in-vitro data, insulin was inactivated by pancreatic enzymes or the supernatants of intestine or liver homogenates. FK-448 suppressed the digestion of insulin by pancreatic enzymes and its enhancement of the intestinal absorption of insulin was found to be related to its inhibition of digestive enzymes, especially chymotrypsin.

There have been many studies on the intestinal absorption of macromolecular substances, most of which have shown that macromolecules, such as some proteins, are poorly absorbed from the intestine except in neonatal rats (Brambell et al 1958; Jones & Waldman 1972; Walker & Cornell 1972; Walker & Isselbacker 1972a, b; Warshaw et al 1974; Hemmings & Williams 1977; Williams & Hemmings 1978).

Oral medication with insulin has been attempted with animals, but has not yet proved practically successful (Arrieta-Molero et al 1982; Patel et al 1982) because insulin is too unstable in the digestive tract. Danforth & Moore (1959) found DFP (diisopropylfluorophosphate) to be effective in inhibiting the digestion of insulin so that some was absorbed from the intestine in small quantities; however, DFP proved too toxic for clinical use.

As insulin is probably degraded by digestive enzymes, especially proteases, in the intestinal ducts, we have examined the protecting effects of some inhibitors of proteolytic enzymes on the digestion in-vitro, and their promoting effects on the intestinal absorption of insulin in-vivo.

MATERIALS AND METHODS

N^{α}-Carbobenzoxy-L-glutamyl-L-phenylalanine(Z-Glu-Phe), N^{α}-carbobenzoxy-L-phenylalanyl-L-arginine-4-methylcoumaryl-7-amide(Z-Phe-Arg-MCA; Barret & Kirschke 1981), pepstatin (Umezawa et al 1970a), leupeptin (Kondo et al 1969) elastatinal (Umezawa et al 1973), bestatin (Umezawa et al 1976), E-64 (*N*-[*N*-(1,3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine) (Hanada et al 1978) and chymostatin (Umezawa et al 1970b) were purchased from Peptide Institute Inc., Osaka, Japan. N^{α}-Benzoyl-L-arginine β-naphthylamide-(BANA), glycyl-L-tyrosine amide acetate (Gly-Tyr-NH_2), N^{α}-benzoyl-D,L-phenylalanine α-naphthylester (Bz-D,L-Phe-O-Nap), *N*-ethylmaleimide (Kirschke et al 1977) and bovine insulin (25 U mg^{-1}) were purchased from Sigma Chemical Co., Ltd. A Glucose B-Test kit (the glucose oxidase method) was purchased from Wako Pure Chemical Industries Ltd, Osaka. *N,N*-Dimethylcarbamoylmethyl-*p*- (*p'*-guanidinobenzoyloxy)phenylacetate methanesulphonate (FOY-305; Tamura et al 1977) was obtained from Ono Pharmaceutical Co., Ltd, Osaka. 4-(4-Isopropylpiperadinocarbonyl)phenyl 1,2,3,4-tetrahydro-1-naphthoate methanesulphonate (FK-448; Fujii et al 1984) was obtained from Tokyo Research Laboratories of Kowa Co., Ltd, Tokyo. Pancreatin was obtained from Mikuni Chemical Co., Ltd, Osaka.

Male, Wistar strain rats (180–230 g), male, ICR strain mice (23–30 g) (Japan Laboric Service Co., Ltd, Kyoto) and male, Beagle Dogs (ca 11 kg) (Hazleton Co., Ltd) were used after withholding their food for 18 h.

Enzymes

Cathepsin A, C (Hirado et al 1981) B, H (Hirado et al 1983) and L (Barret & Kirschke 1981) were

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prepared from rat liver using Sephadex G-200, Sephadex G-75 and/or DEAE-cellulose as described previously. Cathepsin G was prepared from rat neutrophil leukocytes using CM-cellulose by the procedure of Gerber et al (1974).

Pancreatin was used as a source of pancreatic enzymes. 10 g of pancreatin was dissolved in 100 ml of deionized water, stirred at 4 °C for 1 h, and centrifuged at 10 000*g* for 30 min. The supernatant was used as the enzyme solution after determining its protein concentration by the method of Lowry et al (1951).

Supernatants of homogenates of intestine or liver were prepared from rat tissues. For this, rats were decapitated, their intestines and livers collected and washed with saline (0·9% NaCl), and a 20% homogenate in 50 mM sodium phosphate buffer, pH 7·4, was prepared in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 105 000*g* for 60 min and the supernatant was used as the enzyme solution as in the case of pancreatic enzymes.

Effects of some proteolytic enzyme inhibitors on insulin degradation

Effects of some proteolytic enzyme inhibitors on insulin degradation caused by pancreatic enzymes or supernatants of homogenates of intestine or liver were examined. Mixtures of 20 mg protein of the enzymes, 300 μg of insulin and the inhibitors were diluted to 5 ml with 50 mM sodium phosphate buffer, pH 7·4, containing 100 mM NaCl and incubated at 37 °C for 30 min. The mixtures were then cooled to 0 °C, and their residual hypoglycaemic activities were tested by injecting them intraperitoneally into mice and measuring the blood glucose level 1 h later with a Glucose B-Test kit.

The effects of FK-448 on some cathepsin activities

The effects of FK-448 on the hydrolytic activities of some cathepsins were examined as follows (Hirado et al 1981; Hirado et al 1983); enzyme activities are indicated in the parentheses as the hydrolytic activity of each substrate used in the reaction. The incubation of the enzyme reaction was conducted at 37 °C.

Cathepsin A (8·3 nmol min^{-1}): 0·2 ml enzyme was preincubated with 0·05 ml FK-448 and 0·05 ml acetate buffer (250 mM, pH 6·0) for 5 min, and 0·2 ml Z-Glu-Phe (50 mM) was added. The mixture was incubated for 30 min, and then 1 ml Ninhydrin reagent was added and boiled. After adding 2 ml EtOH (60%), A_{506} was observed.

Cathepsins B (40 nmol min^{-1}) and L (84 nmol min^{-1}): 0·01 ml enzyme was preincubated with 0·05 ml FK-448, 0·1 ml dithiothreitol (DTT, 25 mM) and 0·33 ml acetate buffer for 5 min, and 0·01 ml Z-Phe-Arg-MCA (10 mM) was added and incubated for 30 min. After that, 0·5 ml acetic acid (10%) was added to stop the reaction, and the fluorescence liberated (excitation at 380 nm and emission at 440 nm) was observed using Hitachi spectrofluorophotometer 650-10M.

Cathepsin C (20·8 nmol min^{-1}): 0·2 ml enzyme was preincubated with 0·1 ml FK-448, 0·1 ml DTT and 0·1 ml acetate buffer for 5 min. After addition of 0·5 ml Gly-Tyr-NH_2 (2 mM), the mixture was incubated for 30 min, and then the liberated ammonia was determined with 10% Nessler's reagent (at 390 nm).

Cathepsin G (3·3 nmol min^{-1}): 0·1 ml enzyme was preincubated with 0·05 ml FK-448 and 0·33 ml Tris-buffer (0·1 M, pH 8·0) for 5 min and 0·02 ml Bz-D,L-Phe-O-Nap (10 mM) was added. Then the mixture was incubated for 30 min, and after addition of 0·5 ml Fast Garnet (0·6 mg ml^{-1}), A_{520} was observed.

Cathepsin H (20·8 nmol min^{-1}): 0·2 ml enzyme was preincubated with 0·1 ml FK-448, 0·1 ml DTT and 0·1 ml acetate buffer for 5 min, and 0·5 ml BANA was added. After incubating for 30 min, 1 ml colour reagent 1 (mersalyl solution, 4·86 mg ml^{-1}) and 1 ml colour reagent 2 (Fast Garnet 2·25 mg ml^{-1}) was added, and then A_{453} was observed.

Inhibition activity of FK-448 on these cathepsins was calculated from the observation of the remaining enzyme activity in the incubation.

Intestinal absorption of insulin

Rats were anaesthetized with ethylcarbamate (0·9 g kg^{-1}, intraperitoneally), and a front midline incision was made to expose the viscera. A hypodermic needle attached to a syringe containing the test solution was then carefully inserted into the lumen of the jejunum 2 cm under the pylorus. Insulin was dissolved in saline (and 0·1 M HCl if necessary) and injected at 2 ml kg^{-1}. Although most inhibitors tested were soluble in water, chymostatin was insoluble, and so it was dissolved in dimethylsulphoxide (final concentration, 10%). For measurement of blood glucose, samples of 0·2 ml of blood were drawn from the inferior vena cava of rats before, and 1 h after treatment (in the preliminary experiment) or before, 0·5, 1, 1·5, 2, 3 and 4 h after that, and were centrifuged at 3000 rev min^{-1} for 10 min. The plasma glucose concentration of samples was determined as above, and indicated as relative

percents of the blood glucose level at each period compared with that before administration (as 100%).

Enteric-coated gelatin capsules containing insulin and FK-448 were administered orally to the dogs and samples of 1 ml of blood were drawn from the median cubital vein before, and 0·5, 1, 1·5, 2, 2·5, 3, 4 and 5 h after treatment, were centrifuged at 3000 rev min^{-1} for 10 min. The plasma glucose concentration of samples was also determined and indicated as above mentioned. Plasma IRI level was determined by double antibody radioimmunoassay using the Insulin-RIA kit (Dainabot RI Laboratories, Japan).

Statistical analysis

Statistical analysis (Siegel 1956) was performed using Student's *t*-test or the Mann-Whitney's U-test (when the data of each group was not equally dispersed by the *F*-test).

RESULTS

Effects of some proteolytic enzyme inhibitors on insulin degradation

The effects of some proteolytic enzyme inhibitors on insulin degradation by pancreatic enzymes, and by supernatants of homogenates of intestine and liver are shown in Table 1.

Table 1. Effects of some proteolytic enzyme inhibitors (1 mM) on insulin degradation, observed by blood glucose level (mg dl^{-1}) in mice, 1 h after i.p. administration of treated insulin: mean values ± s.e., n = 5, and the residual percentage of insulin activities, calculated from blood glucose levels are indicated in the parentheses.

Inhibitor (1 mM)	Blood glucose level and residual insulin activity (mg dl^{-1}) (%) Pancreatic enzymes	Sup. of homogenate of intestine	Sup. of homogenate of liver
FK-448	4 ± 7 (100)	39 ± 4 (18)	61 ± 10 (9)
Chymostatin	5 ± 5 (100)	10 ± 3 (60)	18 ± 1 (33)
Leupeptin	68 ± 3 (8)	77 ± 8 (6)	21 ± 4 (30)
N-Ethylmaleimide	74 ± 5 (6)	33 ± 2 (21)	21 ± 4 (30)
E-64	87 ± 2 (4)	37 ± 7 (19)	31 ± 5 (23)
Elastatinal	87 ± 8 (4)	81 ± 2 (5)	98 ± 5 (0)
Pepstatin	61 ± 5 (9)	47 ± 4 (14)	41 ± 5 (17)
FOY-305	72 ± 5 (7)	90 ± 2 (4)	94 ± 5 (3)
Bestatin	84 ± 6 (5)	45 ± 6 (15)	53 ± 6 (12)

The residual percentage of insulin activity was calculated as follows: blood glucose levels in mice, 1 h after i.p. administration of 0 (saline), 30 μg (0·75 U) kg^{-1}, 100 μg (2·5 U) kg^{-1}, 300 μg (7·5 U) kg^{-1} insulin; 97 ± 2 mg dl^{-1} (0%), 60 ± 12 mg dl^{-1} (10%), 18 ± 4 mg dl^{-1} (33%), 6 ± 5 mg dl^{-1} (100%), N = 5, respectively.

Sup. = supernatant.

Table 2. Percent change of blood glucose level 1 h after intra-intestinal administration of insulin with or without inhibitors of proteolytic enzymes in rats (mean values ± s.e., n = 4, (*); significantly different ($P < 0·05$) from insulin alone by Mann-Whitney's U-test).

Inhibitor (20 mg kg^{-1})	(%)
Saline control	107 ± 6
Insulin 10 mg kg^{-1}	105 ± 12
with leupeptin	99 ± 8
with elastatinal	98 ± 12
with pepstatin	107 ± 12
with *N*-ethylmaleimide	92 ± 15
with FOY-305	101 ± 4
with E-64	110 ± 6
with bestatin	103 ± 5
with FK-448	61 ± 9 (*)
with chymostatin	66 ± 8 (*)

Values are shown as relative percents of the blood glucose level of 1 h later compared with that before administration (as 100%).

FK-448 inhibited the degradation of insulin by pancreatic enzymes, while pepstatin, *N*-ethylmaleimide, E-64 and bestatin inhibited degradation by supernatants of homogenates of intestine and liver to some extent, and leupeptin inhibited degradation by a homogenate of liver. While chymostatin inhibited the degradation in all cases, especially in the case of pancreatic enzymes, elastatinal and FOY-305 caused little inhibition of insulin degradation.

The effect of FK-448 on the hydrolytic activities of some cathepsins

The effect of FK-448 on the hydrolytic activities of some cathepsins was investigated and it was found that it moderately inhibited cathepsin G (IC50: $1·5 \times 10^{-5}$ M) only.

Intestinal absorption of insulin

Data on the effects of some proteolytic enzyme inhibitors on the intestinal absorption of insulin in rats are shown in Table 2. Among the inhibitors tested, only FK-448 and chymostatin resulted in a decrease in the blood glucose level.

Fig. 1A, B show the dose responses of insulin and FK-448 of intra-intestinal administration in rats. In the presence of FK-448, the decrease in blood glucose level caused by absorbed insulin was statistically significant for intra-intestinal administration.

With dogs, as shown in Fig. 2A, B, a significant decrease of blood glucose level and increase of plasma IRI level were observed relating to the combination of insulin and FK-448 administered orally.

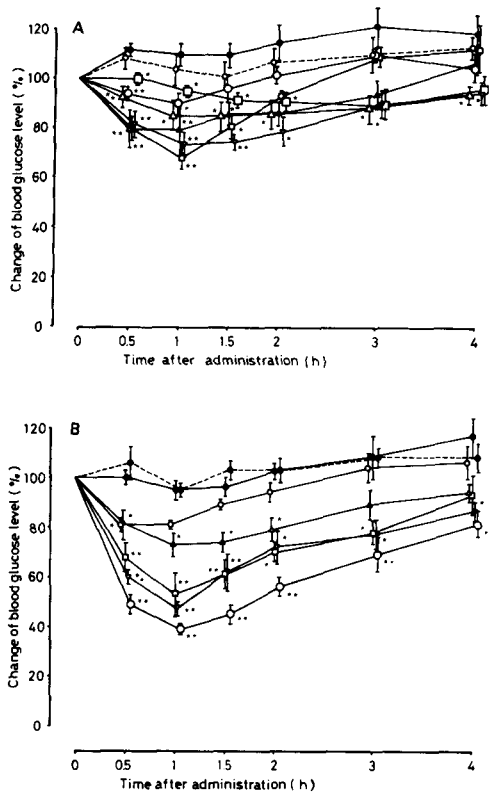

FIG. 1. Percent change of blood glucose level after intra-intestinal administration of insulin with FK-448 in rats. (Mean values ± s.e., n = 6), (*), (**); significantly different ($P < 0.05$, $P < 0.01$, respectively) from saline control by Student's t-test or Mann-Whitney's U-test). (A) Intra-intestinal administration of insulin 2·5 mg (63 U) kg^{-1}. ●—●; Saline control, and ○ - - - ○; insulin 2·5 mg (63 U) kg^{-1} without FK-448, and with FK-448 ○—○; 2·5 mg kg^{-1}, △—△; 5 mg kg^{-1}, □—□; 10 mg kg^{-1}, ▽—▽; 20 mg kg^{-1}, △—△; 40 mg kg^{-1} and □—□; 80 mg kg^{-1}. (B) Intra-intestinal administration of FK-448 20 mg kg^{-1}. ●—●; Saline control, and ● - - - ●; FK-448 20 mg without insulin, and with insulin ○—○ 1·25 mg (31·3 U) kg^{-1}, △—△; 2·5 mg (62·5 U) kg^{-1}, □—□; 5 mg (125 U) kg^{-1}, ▽—▽; 10 mg (250 U) kg^{-1}, and ○—○; 20 mg (500 U) kg^{-1}.

DISCUSSION

Previously we reported that FK-448 was a potent and specific inhibitor of chymotrypsin (Fujii et al 1984). As now shown, FK-448 scarcely inhibited any cathepsins other than cathepsin G (IC50: 1·5 × 10^{-5} M). Moreover, FK-448 is a substance of low toxicity (LD50 value for the rat was greater than 4 g kg^{-1} orally in both males and females, unpublished data).

Among the protease and peptidase inhibitors tested in this work, FK-448 and chymostatin inhibited the degradation of insulin by pancreatic

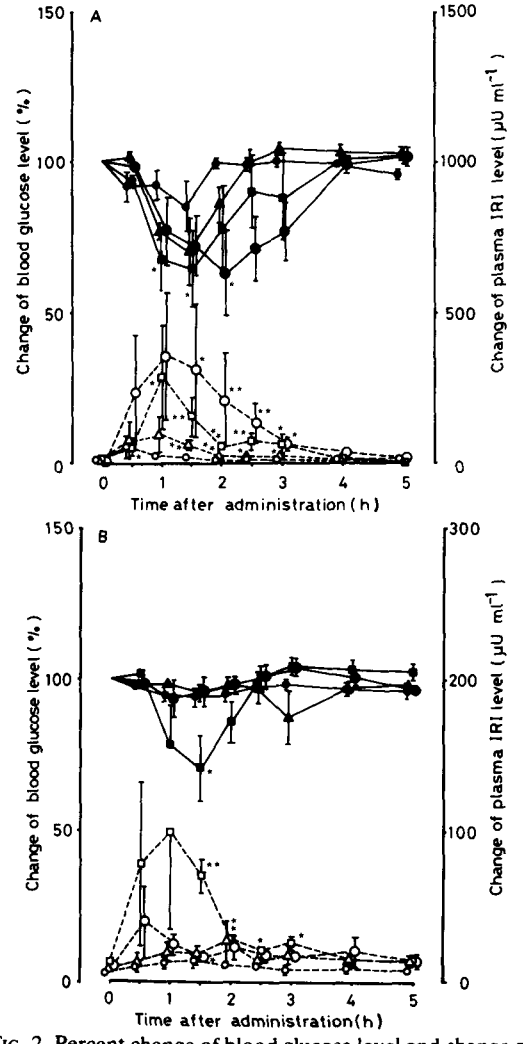

FIG. 2. Percent change of blood glucose level and change of plasma IRI level after oral administration of insulin with FK-448 using entero-coated capsules in dogs. (Mean values ± s.e., n = 6, (*), (**); significantly different ($P < 0.05$, $P < 0.01$, respectively) from the data of insulin 2 mg (50 U) kg^{-1} alone: (blood glucose level; % and IRI level; μU ml^{-1}) 100 and 10·8 ± 2·2 at 0 h, 98·8 ± 1·4 and 8·2 ± 2·2 at 0·5 h, 99·0 ± 1·6 and 10·8 ± 4·1 at 1 h, 98·4 ± 1·4 and 7·8 ± 2·2 at 1·5 h, 98·4 ± 1·7 and 8·2 ± 2·3 at 2 h, 98·2 ± 2·4 and 8·8 ± 2·6 at 2·5 h, 98·2 ± 1·6 and 8·2 ± 3·0 at 3 h, 97·2 ± 1·4 and 11·2 ± 4·1 at 4 h, and 95·4 ± 1·3 and 9·8 ± 2·1 at 5 h (n = 6, mean values ± s.e., respectively) after administration by Student's t-test or Mann-Whitney's U-test. (A) Oral administration of entero-coated gelatin capsule. FK-448 20 mg kg^{-1} with insulin ●—●; 0·25 mg (6·3 U) kg^{-1}, ▲—▲; 0·5 mg (12·5 U) kg^{-1}, ■—■; 1 mg (25 U) kg^{-1} and ●—●; 2 mg (50 U) kg^{-1}. Change of plasma IRI level. FK-448 20 mg kg^{-1} with insulin ○ - - - ○ 0·25 mg (6·3 U) kg^{-1}, △ - - - △; 0·5 mg (12·5 U) kg^{-1}, □ - - - □; 1 mg (25 U) kg^{-1} and ○ - - - ○; 2 mg (50 U) kg^{-1}. (B) Oral administration of entero-coated gelatin capsule. Insulin 0·5 mg (12·5 U) kg^{-1} with FK-448 ●—●; 5 mg kg^{-1}, ▲—▲; 10 mg kg^{-1}, ■—■; 20 mg kg^{-1} and ●—●; 40 mg kg^{-1}. Change of plasma IRI level. Insulin 0·5 mg (12·5 U) kg^{-1} with FK-448 ○ - - - ○; 5 mg kg^{-1}, △ - - - △; 10 mg kg^{-1}, □ - - - □; 20 mg^{-} and ○ - - - ○; 40 mg kg^{-1}.

enzymes. Both inhibitors also enhanced the intestinal absorption of insulin in rats. Chymostatin was also a potent inhibitor of chymotrypsin as described by Umezawa et al (1970b), however, it was not soluble in water. Although some other inhibitors suppressed the degradation of insulin by homogenates of intestine or liver in-vitro, they did not influence the intestinal absorption of insulin. Moreover, FOY-305, a potent trypsin inhibitor (Tamura et al 1977), neither inhibited insulin degradation nor influenced the intestinal absorption of insulin in this study. These results suggest that for enhancement of the intestinal absorption of insulin, degradation of insulin by pancreatic enzymes, especially chymotrypsin, must be inhibited.

Similar results were also obtained by the combined administration of insulin and FK-448 to dogs. In this experiment, plasma IRI levels increased proportionally to the blood glucose levels.

Arrieta-Molero et al (1982) reported that a liposomally bound insulin was probably efficiently absorbed from the intestine via the lymphatic system in diabetic rats and rabbits. However, they also mentioned that the stability and the effectiveness of the liposomes remained unpredictable.

At present, it is not known how macromolecules pass through the intestinal wall into the blood while only a part of them remain active in spite of the protection from digestive enzymes in our method. A large amount of insulin is needed to decrease the blood glucose level. FK-448 is a low toxic substance and is also stable in combination with insulin and the combined administration of insulin and FK-448 may be useful in the clinical treatment of diabetes mellitus.

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