Promotion of Nasal Absorption of Insulin by Glycyrrhetic Acid Derivatives. I

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Nasal absorption of insulin in rats was enhanced by addition of sodium glycyrrhetinate (GA Na), dipotassium glycyrrhizinate (GZ K₂), and carbenoxolone (glycyrrhetic acid hydrogen succinate) disodium salt (GAHS Na₂). The latter agent was the most effective. On addition of 1% GAHS Na₂, plasma immunoreactive insulin levels in rats showed a maximum level of 0.75 mU/ml at 15 min and plasma glucose levels were decreased to about 25 mg/dl after nasal administration of 10 U/kg insulin. In a comparison of the absorption rates of insulin by nasal and intravenous (i.v.) routes in rats, nasal absorptions of 10 U/kg insulin in the presence of 1% GAHS Na₂, 0.5% GA Na and 1% GZ K₂ were 26.5%, 13.2% and 14.5% of that in the case of a 5 U/kg i.v. dose, respectively. Hemolytic activities of GAHS Na₂, GA Na and GZ K₂ were milder than those of Na caprate and Na laurate, and nasal leucine aminopeptidase activity was more strongly inhibited by GAHS Na₂ than by medium chain fatty acid salts, sodium glycocholate, GA Na or GZ K₂. Therefore, it is suggested that GAHS Na₂ is a very useful promoter which does not irritate the nasal mucosal membrane or degrade insulin.

Keywords — insulin; nasal absorption; carbenoxolone disodium salt; sodium glycyrrhetinate; dipotassium glycyrrhizinate; hemolytic activity; leucine aminopeptidase

Introduction

Recently, many investigators have tried to administer insulin using several absorption promoters by nasal,¹⁻³⁾ rectal⁴⁻⁸⁾ and buccal⁹⁾ routes. However, these promoters are not suitable for practical use for medical treatments, because of irritation to the absorptive mucosal membrane. Only ampicillin and ceftizoxime suppositories containing sodium caprate as a promoter are in clinical use in Japan. Glycyrrhetic acid and glycyrrhizinic acid, components of *Glycyrrhiza glabra*, have similar structures to triterpenes and show a surfactant action similar to that of bile acids^{1,6)} or saponins,¹⁰⁾ which are known to be absorption promoters. Therefore, these compounds may act as absorption promoters. Moreover, they show little pseudoaldosterone action, and have anti-inflammatory and anti-allergic properties. They are used in the forms of eyewater and ointment, in cosmetics, and for the treatment of chronic hepatitis. As shown in Chart 1, carbenoxolone is a succinic acid ester of glycyrrhetic acid, and its disodium salt is very soluble in water compared with sodium glycyrrhetinate (GA Na).

In the present study, we investigated the

promoting effect of carbenoxolone, glycyrrhetic acid salt and glycyrrhizinic acid salt on nasal absorption of insulin in rats.

Materials and Methods

Materials — Dipotassium glycyrrhizinate (GZ K₂) and carbenoxolone were gifts from Santen Seiyaku Co. and Maruzen Seiyaku Co., respectively. GA Na was obtained from glycyrrhetic acid (Tokyo Kasei Co.) by treatment

Chart 1. Structures of Glycyrrhetic Acid and Its Derivatives

with sodium bicarbonate. Insulin (23.4 I.U./mg) and L-leucyl- β -naphthylamide hydrochloride were obtained from Sigma Chemical Co. and Nakarai Chemical Co., respectively. *p*-Dimethylaminobenzaldehyde, Insulin C-Test Wako and Glucose B-Test Wako were obtained from Wako Pure Chemical Industries.

Preparation — Insulin test solutions were prepared according to the method described in the previous paper.³⁾

Animal Experiments — Male Wistar rats, 200–300 g, were fasted overnight before experiments and were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg throughout the intravenous, subcutaneous and nasal absorption studies. For the nasal absorption studies, surgical operation was carried out according to the method of Hussain *et al.*¹¹⁾ and insulin solution (0.1 ml/kg) was administered to the rats. For intravenous administration, insulin (5 U/kg) was administered by bolus injection into the femoral vein. For subcutaneous administration, insulin (5 U/kg) was injected under the abdominal skin. Blood (0.5 ml) was taken periodically from the femoral vein into a syringe containing heparin (2.5 U) saline solution and the plasma was separated by centrifugation at 3000 rpm for 10 min. Samples were stored at -20°C until analysis.

Analytical Method — Plasma immunoreactive insulin (IRI) levels were determined by an enzyme immunoassay kit by use of the *o*-phenylenediamine method. Plasma glucose levels were determined by the mutarotase/glucose oxidase method. The decrement of plasma glucose level at each sampling time interval (*D*%) was calculated according to the previous paper.³⁾

Erythrocyte Hemolysis — Erythrocyte hemolysis experiments were performed by a modification of the method of Hirai *et al.*¹²⁾ Human nonfibrinated blood was obtained by centrifugation at 3000 rpm for 10 min at 4°C . After removal of the supernatant solution, the erythrocytes were washed 3 times in 3 ml of physiological phosphate buffer. The washed erythrocytes were suspended in 50 ml of the buffer. Test solutions of promoters were prepared by dissolving them in the buffer at various concentrations. Two milliliters of the test solution was

added to 0.2 ml of the erythrocyte suspension. After incubation for 30 min at 37°C , the mixture was centrifuged at 3000 rpm for 5 min at 4°C , and then the absorbance of the supernatant at 543 nm was measured to calculate the percentage hemolysis. ED_{50} and ED_{100} were calculated from a percentage hemolysis–promoter concentration curve, and hemolysis was expressed as the reciprocal of the concentrations which caused 50% hemolysis and 100% hemolysis.

Determination of Leucine Aminopeptidase Activity in the Nasal Mucosal Homogenate

— After the rats had been anesthetized with ether and decapitated, the nasal mucosa on the septal cartilage was isolated from the frontal bone and homogenized in 10 volumes of cold saline with Phycotron®. Each promoter was dissolved in and adjusted with 0.05 M phosphate buffer, pH 7.0, to various concentrations. Determination of leucine aminopeptidase activity was done according to the method of Hirai *et al.*¹²⁾ and absorbance of the mixture was measured at 450 nm. IC_{50} was calculated from a leucine aminopeptidase activity–promoter concentration curve.

Results

Figures 1 and 2 show plasma IRI and glucose

Fig. 1. Change in Plasma IRI Levels in Rats after Intranasal Administration of Insulin at a Dose of 0 (●), 2 (□), 5 (△) or 10 U/kg (○) in the Presence of 1% GAHS Na_2

Each point represents the mean $\pm$ S.E.M. of 3 to 5 animals.

Fig. 2. Change in Plasma Glucose Levels in Rats after Intranasal Administration of Insulin at a Dose of 0 (●), 2 (□), 5 (△) or 10 U/kg (○) in the Presence of 1% GAHS Na₂

Each point represents the mean $\pm$ S.E.M. of 3 to 5 animals.

levels in rats after nasal administration of various doses of insulin containing 1% carboxoxolone disodium salts (GAHS Na₂).

Peak plasma IRI levels in the presence of GAHS Na₂ were reached at about 15 min irrespective of the insulin dose, and these IRI levels after administration of 2, 5 and 10 U/kg of insulin were 0.10, 0.25 and 0.75 mU/ml, respectively. Decrements of plasma glucose levels in rats were maximum at about 1 h after nasal administration of each insulin dose, and plasma glucose levels then reached about 50 mg/dl after a 2 or 5 U/kg dose, or about 25 mg/dl after a 10 U/kg dose of nasally administered insulin. Amounts of insulin absorbed were nearly proportional to the doses, but decrements of plasma glucose levels were not proportional to the doses. The pharmacological effects may have almost reached saturation after insulin doses from 2 to 10 U/kg in rats. Therefore, we think that 2 U/kg of insulin was enough to lower the plasma glucose levels in rats. On the other hand, plasma glucose level increased with the passage of time on administration of 1% GAHS Na₂ without insulin. A similar increase was observed when the buffer solution was administered to rats.³⁾ The increase in plasma glucose level may be due

Fig. 3. Change in Plasma IRI and Glucose Levels in Rats after Intranasal Administration of Insulin (10 U/kg) in the Presence of 0.1% GAHS Na₂

Each point represents the mean $\pm$ S.E.M. of 3 animals.

to the action of epinephrine and/or histamine secreted owing to stress caused by the surgical operation, etc. Therefore, we concluded that administration of 1% GAHS Na₂ without insulin did not affect insulin or plasma glucose levels.

Figure 3 shows plasma IRI and glucose levels in rats after nasal administration of insulin (10 U/kg) containing 0.1% GAHS Na₂.

In the presence of 0.1% GAHS Na₂, plasma IRI levels were maintained at about 90–110 μ U/ml and plasma glucose levels were decreased to about 35–70 mg/dl from 15 min to 4 h after nasal administration of insulin in rats. It seemed that 0.1% GAHS Na₂ may have acted mildly but continuously on the nasal mucosal membrane.

Table I shows area under the plasma concentration–time curve (0–4 h) (AUC_{0-4}) values of plasma IRI levels and relative decrements of plasma glucose after intranasal administration of insulin with promoters in rats.

The optimum concentration of GAHS Na₂ as an insulin absorption promoter may be considered to be about 1%. At 0.5% GA Na and 1% GZ K₂, the peak times (t_{max}) of plasma IRI levels were 0.5 and 2 h, respectively. The AUC_{0-4} values of plasma IRI levels were 498 h $\cdot$ μ U/ml on addition of GA Na and 544 h $\cdot$ μ U/ml on addition of GZ K₂. The t_{max} value of plasma IRI

TABLE I. Mean Area under Plasma Immunoreactive Insulin Level-Time Curve after Intranasal Administration of Insulin in the Presence of GA Na, GZ K₂ or GAHS Na₂ in Rats

Promoter		Insulin dose (U/kg)	<i>t</i> _{max} (min)	<i>C</i> _{max} (μU/ml)	<i>AUC</i> ₀₋₄ ^{a)} (h · μU/ml)	Bioavailability ^{b)} (%)	<i>D</i> ^{c)} (%)
Compound	Conc. (%)						
None (control)	0	0	—	—	28.7 ± 5.1	0	0
Nasal							
None	0	10	60	67.0 ± 3.4	172 ± 14.2	2.0	3.9 ± 11.0
GA Na	0.5	10	30	229 ± 35.8	498 ± 141	6.6	40.0 ± 7.6
GZ K ₂	1	10	120	187 ± 8.7	544 ± 59.4	7.3	51.7 ± 2.3
GAHS Na ₂	1	2	15	132 ± 36.7	164 ± 27.1	9.5	47.8 ± 2.7
	1	5	15	247 ± 34.5	301 ± 26.6	7.7	54.9 ± 5.3
	1	10	15	744 ± 122	967 ± 93.1	13.2	74.2 ± 2.6
	0.1	10	30	110 ± 0.3	400 ± 20.6	5.2	66.8 ± 4.3
	0.5	10	15	445 ± 99.2	553 ± 62.4	7.4	57.3 ± 2.0
	2	10	15	752 ± 50.8	788 ± 75.4	10.7	65.4 ± 1.9
	1	0	—	—	30.9 ± 2.4	—	5.1 ± 3.5
	—	5	—	—	3576 ± 507	100	81.8 ± 1.0
i.v.	—	5	—	—	3576 ± 507	100	81.8 ± 1.0

a) Each value is the mean ± S.E.M. for 3 to 6 animals.

b) Bioavailability: $\frac{AUC_{(nasal)} - AUC_{(control)}}{AUC_{(i.v.)} - AUC_{(control)}} \times \frac{\text{dose (i.v.)}}{\text{dose (nasal)}} \times 100$

c) *D* is relative decrement of plasma glucose levels.

levels for GAHS Na₂ was 0.25 h without 0.1% GAHS Na₂.

Relative decrements of plasma glucose levels were 40% on addition of 0.5% GA Na and 51.7% on addition of 1% GZ K₂. *AUC*₀₋₄ after intranasal insulin administration was approximately 13% in the presence of 1% GAHS Na₂ compared with *AUC*₀₋₄ following an intravenous dose. Notably, the addition of 0.1% GAHS Na₂ produced a value of 66.8% for *D*% in spite of the low insulin absorption (5.2%). It seemed that the increase in *AUC*₀₋₄ was not proportional to

insulin dose above 5 U/kg in rats. A possible saturation of degradation of insulin by protease(s) in nasal mucosa may account for this. Thus, the addition of 1% GAHS Na₂ increased the bioavailability of insulin 10 U/kg to about twice that of insulin 5 U/kg. This was because the plasma IRI levels were maintained at about 100 μU/ml from 30 min to 4 h after intranasal administration of insulin in rats.

We studied the hemolytic activity and inhibition of leucine aminopeptidase activity to examine possible mechanisms of absorption promo-

TABLE II. Effect of Promoters on Erythrocyte Hemolysis

Promoter	Hemolysis (ml/mg)	
	ED ₅₀	ED ₁₀₀
GAHS Na ₂	0.34	0.08
GA Na	1.10	0.04
GZ K ₂	0.02 >	0.02 >
Na caprylate	0.11	0.06
Na caprate	0.74	0.67
Na laurate	5.62	5.0
Na glycocholate	0.08	0.05

TABLE III. Inhibitory Effect of Promoters on Leucine Aminopeptidase Activity in Rat Nasal Mucosa

Promoter	IC ₅₀ (mM)
GAHS Na ₂	0.064
GA Na	5.338
GZ K ₂	1.079
Na caprylate	43.747
Na caprate	10.911
Na laurate	34.388
Na glycocholate	1.743

tion by these promoters. The effects of the promoters on erythrocyte hemolysis are shown in Table II.

Hemolytic activity of GZ K₂ was weaker than that of Na caprate or Na laurate, and the activity of GAHS Na₂ was similar to those of Na caprylate and Na glycocholate. GZ K₂ did not show 100% hemolysis at a concentration below 5%. Hemolytic activity of GAHS Na₂ and GA Na depended very much on concentration between 50% hemolysis and 100% hemolysis, but that of fatty acids and Na glycocholate did not. Therefore, GAHS Na₂, GA Na and GZ K₂ may act mildly on the nasal mucosal membrane.

The 50% inhibitory effects of absorption promoters on leucine aminopeptidase activity in the rat nasal mucosa are shown in Table III.

This peptidase activity was strongly inhibited by GAHS Na₂, GA Na, GZ K₂ and Na glycocholate but not by fatty acid salts. In particular, GAHS Na₂ inhibited this enzyme at a very low concentration. These results suggest that GAHS Na₂ is a very useful promoter that does not irritate the nasal mucosal membrane or degrade insulin.

Discussion

In this study, we investigated the promoting effects of glycyrrhetic acid derivatives on nasal absorption of insulin in rats. We found that these substances significantly promoted absorption of insulin through the nasal mucosa in rats.

When insulin (10 U/kg) containing 1% GAHS Na₂ was administered to the nasal cavity of rats, the peak plasma IRI level appeared at 15 min and its AUC_{0-4} was 967 h · μ U/ml. We reported³⁾ that t_{max} of insulin in the presence of 1% sodium caprate was 5 min and its AUC_{0-4} was 1419 h · μ U/ml. Therefore, t_{max} of insulin levels in the presence of GAHS Na₂ was longer than that in the presence of sodium caprate, and the insulin bioavailability was inferior. However, at this insulin dose, the decrements of glucose levels were not very different between sodium caprate and GAHS Na₂.³⁾

On nasal administration of 2 and 5 U/kg insulin, the AUC_{0-4} values and relative decrements of plasma glucose were not very different be-

tween GAHS Na₂ and sodium caprate. Thus, these results show that GAHS Na₂ has as strong a promoting action as sodium caprate.³⁾

Interestingly, plasma IRI levels were kept at about 100 μ U/ml from 30 min to 4 h after nasal administration of insulin (10 U/kg) containing 0.1% GAHS Na₂. This maintenance of plasma IRI levels led to a significant decrease in plasma glucose. In practice, the AUC_{0-4} value of insulin after nasal administration of 2 U/kg insulin containing 1% GAHS Na₂ was not significantly different from that of insulin after nasal administration of 10 U/kg insulin alone, but the decrement of plasma glucose was greater after administration of 2 U/kg insulin containing 1% GAHS Na₂ than after 10 U/kg insulin alone. The maximum IRI level exceeded 100 μ U/ml after administration of 2 U/kg insulin containing 1% GAHS Na₂. These results indicate that decrease in plasma glucose is caused by at least 100 μ U/ml insulin in plasma. Therefore, in spite of the relatively low absorption rate of insulin in the presence of 0.1% GAHS Na₂, its pharmacological action was as great as that in the case of 1% GAHS Na₂. Almost all promoters show only a transient effect on absorption of drugs. It seems that this phenomenon is caused by homeostasis of the mucosal membrane. However, 0.1% GAHS Na₂ may act mildly but continuously on a biomembrane, and this mechanism of action on the biomembrane by 0.1% GAHS Na₂ should be studied in detail.

The low hemolytic activity of glycyrrhetic acid and its derivatives indicates that these compounds do not cause acute irritation to the nasal mucosal membrane. Moreover, these compounds have been used as external drugs for dermal inflammation.^{13,14)} Therefore, they are not likely to cause edema by nasal administration. We think that these compounds are likely to be safer for use on membranes than other absorption promoters.

It is considered that drug absorption routes through mucosal membranes are transcellular and/or paracellular.¹⁵⁾ Insulin may be absorbed mainly through the paracellular route because of its hydrophilicity and high molecular weight. However, if insulin is absorbed through the mucosal membrane by the transcellular route,

insulin may avoid a first-pass effect due to its degradation by nasal mucosal leucine aminopeptidase, which is inhibited by glycyrrhetic acid and its derivatives.

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