Nasal Insulin Delivery in Rabbits Using Soybean-Derived Sterylglucoside and Sterol Mixtures as Novel Enhancers in Suspension Dosage Forms

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The effect of a soybean-derived sterol mixture (SS) and a steryl glucoside mixture (SG) as enhancers of the nasal absorption of insulin in rabbits was investigated. SS consists of β-sitosterol (Sit), campesterol (Camp), stigmasterol (Stig) and brassicasterol (Bras), and SG is a mixture of their monoglucosides. For each component of SS tested for efficacy in promoting the systemic absorption of nasally administered insulin, the following order was observed: Sit>Camp>Stig. This finding was in agreement with the order of the enhancers' lipophilicity. In the case of SG, the effect of β-sitosterol β-D-glucoside (Sit-G) was significantly greater than that of SG. The pharmacological bioavailability was 6.7% for SG and 11.3% for Sit-G in the suspension dosage forms. SG showed a greater degree of enhancement of insulin permeation through the nasal mucosa than SS. To elucidate the contribution of SG to the enhanced absorption, insulin permeation through an artificial membrane and the nasal mucosa was investigated in vitro, and the results were compared with those for SS. The findings suggest that SG and SS have some effect on nasal mucosa lipids.

Key words: insulin; nasal administration; soybean-derived sterylglucoside; soybean-derived sterol; suspension dosage form; bioavailability

Interest in the drug delivery of peptides and proteins via nonparenteral routes has recently been increasing. Presently, most peptides and proteins are not effective when administered orally, so they must be administered by intravenous or dermochysis injections which are poorly accepted by most patients. The nonparenteral routes that have been investigated for insulin delivery include the nasal1–4,5 buccal,6 rectal,6 ocular,7) and pulmonary routes.8) Results indicate that the nasal route is the most promising. In an attempt to increase the bioavailability of insulin after nasal administration, a number of enhancers have been investigated. Among them, sodium glycolate,9 sodium taurodihydrofusidate10 and didecanoyl-l-α-phosphatidylcholine11 were effective enhancers of the nasal absorption of insulin but were also reported to alter nasal morphology.12,13

We have already reported the effect of a soybean-derived sterylglucoside mixture (SG) and its aglycon (SS) as an enhancer for the nasal absorption of insulin in rabbits.14,15) These compounds are natural products and have been shown to induce no histological changes in nasal tissue.16) SG is not soluble in water and only slightly soluble in peanut oil, but possesses excellent properties as an enhancer in suspension, i.e., a phosphate-buffered saline solution and peanut oil,14,15 and in powder dosage forms. It is better than the well known absorption enhancer, sodium glycolate.

The purpose of this study was to evaluate the effect of SS and SG as enhancers of the nasal absorption of insulin. In addition, the mechanism of enhanced insulin absorption by SG was investigated by measuring the extent of insulin permeation through an excised nasal mucosa, and measuring the circular dichroism of an insulin solution containing SG.

MATERIALS AND METHODS

Chemicals Bovine insulin (25.7 IU/mg) was purchased from Sigma Chemical Co. (U.S.A.). SG and SS were kindly supplied by Ryukakusan Co., Ltd. (Tokyo, Japan). SG is a mixture of the glucosides of β-sitosterol (Sit, 49.9%),

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Campesterol (Camp, 29.1%), stigmasterol (Stig, 13.8%), and brassicasterol (Bras, 7.2%) as shown in Fig. 1. SS was obtained by hydrolysis of the glucoside bonds of SG components, i.e., SS is the aglycon of SG. β-Sitosterol-β-D-glucoside (Sit-G) was synthesized from glucose and β-sitosterol (mp 298.4°C).17 The components of SS were purchased from Tama Biochemical Co., Ltd. (Tokyo, Japan). Sodium glycolate (NaGC) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

Preparation of Suspension Dosage Forms SS and insulin were passed through a 200 mesh sieve. The insulin suspension was prepared by suspending 40 mg insulin, with or without 1% (w/w) SG or SS, in 10 ml pH 7.3 phosphate-buffered saline solution (PBS) with stirring. The solubility of insulin, SG and SS in PBS is 1.39 mg/ml, 53.42 μg/ml and very low, respectively at 37°C. This suspension dosage form consists of a suspension of insulin and enhancer in PBS.

Administration Methods Female Japanese rabbits weighing between 2.5 and 3.0 kg (Saitama Experimental Animal Supply Co., Japan) were fasted for 24 h before drug administration. A 10.0 cm polyethylene tube with a diameter of 1.05 mm (Natsume Seiskusho, Tokyo, Japan) was fitted to a syringe and inserted into the nose of a rabbit.15 A 250 μl dose (e.g., 10 IU/kg) of the suspension dosage form was loaded into the syringe and administered via the tube into the nasal cavity of a rabbit. There was a wash out period of at least 1 week between treatments.

Evaluation of Pharmacological Bioavailability A 1 ml blood sample was collected serially from the ear vein. Plasma glucose was assayed by the glucose oxidase method using the glucose B-test Wako (Wako Pure Chemicals Ind., Ltd.). The serum glucose level at time-zero was taken as 100%. The area above baseline levels was not included in the counting. The absolute pharmacological availability was expressed in terms of the area under the curves of the total decrease in glucose reduction time (AUG) from 0 to 6 h, calculated using the trapezoidal method.14 It was calculated ac-
Fig. 1. Chemical Structures of Components of a Soybean-Derived Sterylglucoside Mixture (SG)

Numbers in parentheses represent the ratio in SG.

cording to the following equation after i.v. administration of 0.5 IU/kg insulin:

\[
\text{pharmacological bioavailability (D)} = \frac{(AUG_{i,v}/\text{dose}_{i,v})}{(AUG_{i,v}/\text{dose}_{i,n})}
\]

where subscripts represent intravenous (i.v.) and intranasal (i.n.) administration.

Circular Dichromism Studies A CD spectropolarimeter (JASCO Model J600, Japan Spectroscopic Co., Ltd., Tokyo, Japan) was used to illustrate the gradual changes produced by insulin dissociation. i.e., change from hexameric to dimeric and monomeric forms in the presence of various enhancers. Insulin solutions in PBS containing 0.5 g/l zinc insulin saturated with SG (0.092 mmol/l) or 2.5 to 20 mmol/l NaGC were scanned from 300 to 250 nm at a scanning speed of 10 nm/min at 25 °C. A 10 mm pathlength quartz cuvette was used to obtain optimum resolution of the CD spectra. The generated ellipticity values were subsequently converted to molar ellipticities for the entire wavelength range using a computer and the equation \( [\theta]_\lambda = \frac{\theta_\lambda}{(C \cdot l)} \) (where \( \theta_\lambda \) is the observed ellipticity at wavelength \( \lambda \), C is the decimolar insulin concentration, and l is the pathlength in decimeters).

Insulin Permeation Study To prepare the lipid artificial membrane, an RS membrane filter (RS type, Sartorius Co., Ltd.) was soaked with lipids, a mixture of n-caprylic acid and lauryl alcohol, (4 : 0.92), at 30°C for 5 min. Three types of membrane, i.e., artificial membranes, with or without lipids, and the excised nasal mucosa were mounted in two-chamber diffusion cells at 37°C. Six milliliter of insulin suspension (4 mg insulin/ml, C_0) in PBS containing 1% SG or SS was used as the donor solution and PBS as the receiver solution.

The insulin concentration in the receiver solution (C_0) was determined using HPLC or EIA (Dinabot Co. Ltd., Japan). The HPLC method used has been described previously by Nakazawa and Nagase. The C_0 values were plotted as a function of time (t) from 0 to 3 h. The permeability coefficient (K) was calculated according to the following equation:

\[
J = (dC/dt) \times V/S = K \times C_0
\]

where \( J \) is the flux, \( S \) denotes the area of the nasal mucosa available for permeation (0.503 cm²), and \( V \) is the volume of the receiver solution (6 ml).

HPLC Conditions for SS and SG The equipment consisted of a Shimadzu pump; an SDP-6AV UV spectrophotometer (Shimadzu, Kyoto, Japan); a C-R6A chromatopack; and a YMC C-18 column (ODS-2, 5 mm, 4.6×150 µm). The mobile phase was CH₃OH at a flow rate of 0.5 ml/min at 40°C and detection was at 210 nm.

Statistical Analysis Data from the animal experiments were compared by analysis of variance and Student's t-test. A p-value < 0.01 or 0.05 was considered significant.

RESULTS

Effect of Components of SS and SG at a 1% Concentration on Insulin Absorption 1% SG exhibits its maximum effect on insulin absorption in an insulin suspension in PBS. Therefore, 1% concentrations of SS, SG, and their components were used to compare their enhancement of insulin absorption.

Figure 2 shows the change in glucose concentration after administration of insulin containing 1% SG or Sit-G. The minimum plasma glucose level was observed 1–2 h after administration, and the hypoglycemia continued for 6 h after administration.

Figure 3 shows the pharmacological bioavailability after nasal administration of insulin suspensions containing 1% of each component of SS or SG. Only the insulin solution in PBS as a control was not absorbed. One of the components of SG, Sit-G, significantly promotes the nasal absorption of insulin when compared with the effect of SG (p<0.05), and it was most effective in producing hypoglycemia among all the enhancers studied. The AUG was 216.9±33.9%·h for 1% Sit-G and 127.8±31.1%·h for 1% SG. In SS, the rank order of effectiveness in producing hypoglycemia was: SS ≈ Sit-G ≈ Camp-G ≈ Stig. Sit-G also induces hypoglycemia to a greater extent than its aglycon, Sit (p<0.05). The pharmacological bioavailability of SS was 5.3%, and the calculated bioavailability was 4.6% when that of each component of SS was combined, in the ratio of the components in SS.

The relationships between the HPLC retention time and the pharmacological bioavailability of each component of SS were examined. The order of the enhancement effect was in agreement with the order of lipophilicity of the SS components (retention times, Sit: 20.5 min, Camp: 18.6 min, Stig: 17.0 min).

Interaction of SG with Insulin The interaction of SG with insulin was clarified by circular dichroism. The effects
Fig. 2. Plasma Glucose Level after Nasal Administration of Insulin (10 IU/kg) in PBS Containing 1% SG (●) or 1% Sit-G (○), and Control (□).
Each value represents the mean±S.D. (n=3–4). * p<0.05, ** p<0.01 versus control.

![Graph showing change in plasma glucose level over time](image)

Fig. 3. Pharmacological Bioavailability of Nasally Administered Insulin (10 IU/kg) in PBS Containing 1% of Each Component of SS or SG.
Each value represents the mean±S.D. (n=3–4). * p<0.05, ** p<0.01.

![Graph showing pharmacological bioavailability](image)

of the incorporation of SG and NaGC on the magnitude of the insulin band are shown in Fig. 4. The gradual addition of NaGC to the insulin solution caused a progressive reduction in molecular ellipticity, but SG did not.

**Effect of SG and SS on Nasal Mucosa Lipids** To elucidate the contribution of SS and SG to the enhancement of insulin absorption, insulin permeation through the artificial membranes (with or without lipids), and excised nasal mucosa was investigated *in vitro*. Figure 5 shows the $K$ values of insulin through each membrane. In the case of the artificial membrane (AF) alone, it was observed that the permeation of the insulin control solution was almost the same as that of the solution containing 1% SG while that of the solution containing 1% SS decreased.

In the case of the artificial membrane with lipids (AFL) and nasal mucosa, no permeation of insulin was observed in the controls. However, the degree of insulin permeation increased significantly on addition of both SG and SS to the solution. The ratio of the $K$ values of SS and SG was similar for the artificial membrane, with and without lipids, and nasal mucosa. This result showed that insulin permeation through the artificial membrane with lipid and nasal mucosa may have been prevented by lipids in the membrane. SG showed a significantly higher enhancement of insulin permeation than SS ($p<0.001$).

**DISCUSSION**

Among the components of SS those with a higher lipophilicity had a greater enhancing effect on the absorption of insulin. The most hydrophobic compound in SS, Sit, is the most effective enhancer among the components of SS. This result suggests that the affinity of SS for lipids in the nasal mucosa may be one of the important factors in the enhancement of absorption. SS and SG may have different enhancing mechanisms on nasal absorption, because Sit-G is more hydrophilic than Sit, but has a greater enhancing effect than Sit on the absorption of insulin. Sit-G, with a higher lipophilicity among the components of SG, may have a greater enhancing effect on the absorption of insulin than SS.

Generally, enhancers affect mainly (1) drugs, (2) peptidases and mucus at the mucosa surface, and (3) mucus; (1) SG and SS are not soluble in water and did not disperse in-
insulin to monomers as did NaGC which is known to prevent the self-association of insulin as shown by circular dichroism. Our findings suggest that the deggregation of insulin by SG appears to be of minor importance in insulin nasal absorption enhancement.

(2) SG and SS might affect peptidases on the nasal mucosa. However, it was difficult to examine the change in stability of insulin because SS and SG are very poorly soluble in PBS.

(3) From the insulin permeation studies, the ratio of the K values of SG and SS, \(K_{SG}/K_{SS}\), was 2.17 for the lipid artificial membrane and 2.06 for the nasal mucosa. These values were very similar suggesting that SG and SS may affect mucosal lipids.

SG appears to have a higher affinity for lipid in mucosae because of the low solubility in PBS, suggesting that SG interacts directly with lipid in the nasal mucosae. The glucose group of SG may contribute to the enhancement, although free glucose does not (data not shown). This was also supported by the finding that Sit-G showed a significantly higher degree of enhancement than Sit (Fig. 3). SG and SS had no effect on the buccal mucosa which is covered with stratum corneum (data not shown). This result suggests that the sterol components of SG may affect the hydrophobic region of the mucosa and that the glucose group of SG may affect other regions such as the hydrophilic mucus. Pilion et al. reported some differences in the enhancement of insulin absorption when two different glycosides were linked to the same alkyl chain. The results of this report support the hypothesis that the glucose component of SG contributes to the enhancement of insulin absorption.

It was clear that SG affects lipids, however, no histological change was observed in the nasal mucosa after a single and subchronic administration of insulin in powder dosage form containing SG. The nasal mucosa recovered less than 60 min after administration. These findings suggest that the effect of SG is transient.

The efficacy of SS components in promoting the systemic absorption of nasally administered insulin, was clarified: SitCG > SitG, being in agreement with the order of the enhancers' lipophilicity. The pharmacological bioavailability was 6.7% for SG and 11.3% for one of the components of SG, Sit-G. SG induced a greater degree of hypoglycemia than SS. This may be because of the contribution of the glucose residue. It was clear that the mechanism of enhancement of insulin absorption by SG had a transitory effect on mucosal lipids, but not on insulin.

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REFERENCES