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Effects of a sperminated gelatin on the nasal absorption of insulin

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Abstract

The effects of a sperminated gelatin (SG), which was prepared as a candidate absorption enhancer by the addition of spermine to gelatin, on the nasal absorption of insulin, were examined in rats. The AUC of immuno-reactive insulin levels in the plasma after nasal administration of insulin were increased 5.3-fold by addition of 0.2% SG, and the plasma glucose levels fell in a manner dependent on the insulin levels. In Calu-3 cell monolayer permeation experiments, SG showed significant enhancing effects on 5(6)-carboxyfluorescein (CF), FITC-dextran (MW 4400, FD4) and insulin. Evaluation of the tight junctions in the Calu-3 cell monolayers based on the Renkin molecular sieving function suggests that the pore occupancy/length ratio of the permeation pathways for water-soluble molecules in the tight junctions increases, while the equivalent cylindrical pore radius is not changed by SG treatment. SG may transform the true tight junctions, which act as a barrier for water-soluble molecules, into pathways for CF and FD4 to increase their number. SG is a good candidate for a safe absorption enhancer to produce a slight modification of the permeability of the paracellular pathway of mucosal membranes, while retaining the sieving property of the epithelial membranes.

Keywords: Nasal administration; Insulin; Spermine; Gelatin; Renkin function

1. Introduction

Nasal administration of peptide and protein drugs is a useful way to avoid the problems associated with parenteral formulations, such as tissue invasion, and also to improve patient compliance. The use of absorption enhancers and proteolytic enzyme inhibitors and suitably designed formulations are all useful approaches to increase the bioavailability of peptide and protein drugs in novel delivery systems (Davis and Illum, 2003; Morimoto et al., 1995; Dondeti et al., 1996). The absorption enhancers, which increase the permeability of drugs through the epithelial membranes without causing any tissue damage, are especially useful for the delivery of peptide and protein drugs. Although surfactants, bile salts and fatty acids have been evaluated as absorption enhancers, and most of them exhibit permeation-enhancing effects to produce some form of membrane damage (Marttin et al., 1995; Merkus et al., 1993). Most of the candidate enhancers reported are able to open the tight junctions of epithelial cell layers to suppress transepithelial electrical resistance (TER) and increase the paracellular permeability of drugs, such as mannitol and FITC-dextrans (Davis and Illum, 2003). The mechanism of permeation-enhancement needs is to be well understood in order to develop safer absorption enhancers.

It has recently been reported that cationic polymers, including chitosan and its derivatives (Illum et al., 1994; Aspden et al., 1996), poly-L-arginine (Natsume et al., 1999; Miyamoto et al., 2001; Ohtake et al., 2003) and aminated gelatins (Seki et al., 2005), are able to improve the absorption of peptide and protein drugs through mucosal membranes while causing negligible damage to these membranes. The cationic polymers could interact with the luminal surface of mucus membranes directly by an ion-ion interaction and then induce signals that would open tight junctions resulting in intercellular permeation of water-soluble drugs (McEwan et al., 1993).

In this study, the effects of a sperminated gelatin (SG), which was prepared as a candidate absorption enhancer by the addition of spermine to gelatin (Seki et al., 2006), on the

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nasal absorption of insulin were examined in rats. In addition, the permeation characteristics of the paracellular pathway of Calu-3 cell monolayers were examined based on the Renkin molecular sieving function (Renkin, 1954; Hosoya et al., 2004). The equivalent cylindrical pore radius (R) and pore occupancy/length ratio (ε/L) of the layers were calculated from the apparent permeability coefficients ($Papp$) and the diffusion coefficients (D) of 5(6)-carboxyfluorescein (CF) and FITC-dextran (MW 4400, FD4), and the permeability coefficients of insulin calculated using the Renkin function were compared with the observed values to evaluate the enzymatic degradation of insulin during the transport process (Seki et al., 2006).

2. Materials and methods

2.1. Materials

Gelatin (isoelectric point = 9.0, MW 100 kDa) was kindly supplied by Nitta Gelatin Co. Ltd. (Osaka, Japan). Recombinant human insulin (28.7 IU/mg), FD4 and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MA, USA). Glucose B-test kits and spermine tetrahydrochloride were purchased from Wako Pure Chemical Industries (Osaka). CF and 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) were purchased from Acros Organics (NJ, USA) and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals were of reagent grade and used as received.

2.2. Synthesis of SG

Gelatin was reacted with spermine to obtain SG in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride by a method previously reported (Seki et al., 2006). In brief, gelatin (10 g) was dissolved in 0.1 M phosphate buffer (pH 5.0, 250 mL), spermine tetrahydrochloride (32.4 g, 0.093 mole) was added to the solution and then the pH of the solution was adjusted to 5.0 with hydrochloric acid. The resulting solution was mixed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (5.35 g) and the total volume was adjusted to 500 mL by addition of phosphate buffer (pH 5.0). The reaction was allowed to take place at 37 °C for 18 h. The resulting SG was purified by dialysis for 48 h then the powder was obtained by lyophilization.

In order to determine the amino group content in the SG, 1 mL gelatin or SG solution (0.50 mg/mL) in phosphate buffered saline (PBS, pH 7.4) was mixed with 1.0 mL sodium bicarbonate solution (4.0%) and 1.0 mL TNBS solution (0.10%). The mixture was kept at 40 °C for 2 h protected from light and then the absorbance of the solution at 415 nm was determined (Wang et al., 2002). A calibration curve was prepared using β-alanine. The primary amino group content (PA) was expressed as the amount of TNBS-reactive amino groups in 1 g gelatin or SG. Since the addition of spermine introduced not only primary amino groups but also secondary groups to the gelatin, the total amino group content (TA) of SG was calculated using the following Eq. (1):

$$TA = (PA_{SG} - PA_{native}) \times 3 + PA_{native} \quad (1)$$

where PA_{SG} and PA_{native} are the primary amino group contents of SG and native-gelatin, respectively.

2.3. Nasal administration of insulin

Animal experiments were carried out in accordance with the Guiding Principles for the Care and Use of Experimental Animals, Hokkaido Pharmaceutical University (1998). Male Wistar rats (Sankyo LaboService Co.), weighing 210–260 g, were fasted for 24 h before the experiments, but had free access to water. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg. The rats were then placed in the supine position, a tracheal cannulation was performed to maintain respiration, and the trachea leading to the nasal cavity was ligated to prevent the transuding liquid oozing from the surgical incision. Another polyethylene tube, with a closed top, was inserted through the oesophagus to the posterior part of the nasal cavity to prevent drainage of the applied drug solution into the nasopharynx. The insulin solutions for administration were prepared as follows: insulin (1.0 mg) was dissolved in 0.01 M hydrochloric acid (100 μL) and then the solution was mixed with PBS (43.5 μL) to give 200 IU/mL insulin solution. The solution was mixed with the same volume of SG solution (0–0.40%) in PBS and then the resulting solutions were administered to the nasal cavity through the nostrils using a pipette (50 μL/kg for each cavity, total 100 μL/kg). The dose of insulin was 10 IU/kg. Blood samples of 0.2 mL were withdrawn from the femoral vein 10 min before administration and at predetermined times after dosing for up to 5 h. After centrifugation of blood samples at 10,000 rpm for 5 min, the plasma was isolated. The plasma glucose concentration was determined using a glucose B-test kit (glucose oxidase method) and the immuno-reactive insulin (IRI) in the plasma was determined using an enzyme immunoassay kit (YKO 60 Human Insulin EIA kit, Yanaihara Institute, Shizuoka, Japan) according to the manufacturer's instructions.

2.4. Nasal administration of CF

Experimental animals were prepared according to the same procedure for the insulin administration. CF solution (0.4%) in PBS was applied to the nasal cavity (0.4 mg/kg) and then blood samples of 0.25 mL were withdrawn from the femoral vein for up to 8 h. The CF concentration in plasma was determined using a spectrofluorometer (F-2000, Hitachi, Tokyo, Japan) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

2.5. Calu-3 cell monolayer permeation experiments

Calu-3 cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 40 μg/mL gentamicin and 1% nonessential amino acids, in a humidified atmosphere of 95% air

and 5% CO_2 at 37 °C. Cells from passage number 43–63 were seeded (4.5×10^5 cell/cm^2) on polyester filter inserts (pore size 0.4 μm, area 0.33 cm^2, Transwell, Costar) and cultivated in the medium for 9–15 days before starting the drug transport experiments. Simultaneous transport of FD4, CF and insulin through the confluent Calu-3 cell monolayers was observed at 37 °C. CF (30 μg/mL), FD4 (5.0%), insulin (100 IU/mL) and SG (0 or 0.20%) were dissolved in PBS and the solution (100 μL) was applied to the apical side. Hanks balanced salt solution (HBSS, pH 7.4, 0.60 mL) was used as the basolateral side solution and this was changed every 30 min for 2 h. TER was measured using Millicell®-ERS (Millipore, MASS, USA) before and after the transport experiments. HBSS was used as the apical and basolateral solutions.

FD4 and CF in the medium were isolated and determined by a gradient HPLC system (Shimadzu, Kyoto, Japan) consisting of a pump (LC-10AT), oven (CTO-6A), detector (RF-10A) and integrator (CR-5A). Separation was carried out using an analytical column (Shodex Asahipak NH2P-50 4E, 250 mm × 4.6 mm i.d.; Showa Denko, Kawasaki, Japan) and a gradient of 0.1 M triethanolamine + 10% acetonitrile–0.1 M triethanolamine + 10% acetonitrile + 60 mM NaCl changing from 30:70 to 80:20 over 30 min. The flow-rate of the eluent was 1 mL/min, the oven was operated at 45 °C and the detector was operated at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The insulin concentration in the medium was determined using the enzyme immunoassay kit.

The *Papp* of penetrant *i* ($Papp_i$) was calculated using the following Eq. (2):

$$Papp_i = \frac{\Delta M_i/\Delta t}{AC_{0,i}} \tag{2}$$

where $(\Delta M_i/\Delta t)$ is the rate of the penetrant *i* appearing on the receiver side, *A* is area of the monolayers and $C_{0,i}$ is the initial concentration of *i*.

The Renkin function (3) was used for characterization of the Calu-3 cell monolayers:

$$F\left(\frac{r_i}{R}\right) = \left(1 - \left(\frac{r_i}{R}\right)\right)^2 \times \left[1 - 2.104\left(\frac{r_i}{R}\right) + 2.09\left(\frac{r_i}{R}\right)^3 - 0.95\left(\frac{r_i}{R}\right)^5\right] \tag{3}$$

where r_i is the molecular radius of penetrant *i* which can be calculated from D_i as the Stokes–Einstein radius. Since CF and FD4 were not metabolized during the permeation process, the *Papp* of CF and FD4 ($Papp_{CF}$ and $Papp_{FD4}$) were used for the calculation to obtain *R* and ε/L as the characteristic parameters for each monolayer using the following Eqs. (4) and (5) and diffusion parameters in Table 1.

$$Papp_{CF} = (\varepsilon/L)D_{CF}F\left(\frac{r_{CF}}{R}\right) \tag{4}$$

$$Papp_{FD4} = (\varepsilon/L)D_{FD4}F\left(\frac{r_{FD4}}{R}\right) \tag{5}$$

Table 1
Diffusion coefficient (D_i) and Stokes–Einstein radius (r_i) of CF, FD4 and insulin at 37 °C

	MW	D_i[a] (cm/s) × 10^6	r_i (nm)
CF	376.3	5.87	0.556
FD4	4400	2.39	1.37
Insulin	5807.6	1.14	2.86

[a] Hosoya et al., 2004.

2.6. Statistical analysis

The statistical significance of each treatment was evaluated by the unpaired Student's *t*-test for two samples and the Dunnett test for multi-samples. StatView software (Ver.5.0, SAS Institute Inc.) was used for the calculations.

3. Results

3.1. Synthesis of SG

SG was prepared from gelatin and spermine using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. The primary amino group content determined as the number of TNBS-reactive amino groups was 0.660 mmol/g. This value was about 2.6 times higher than that of native gelatin (0.255 mmol/g). Since the addition of one spermine molecule transferred three amino groups to the gelatin, the calculated total amino group content of SG was 1.47 mmol/g.

3.2. Nasal absorption of insulin

The effects of SG on the nasal absorption of insulin were examined in rats. The absorption of insulin was detected first by measuring its hypoglycemic effect, i.e. the change in plasma glucose. Fig. 1 shows the effects of SG on the plasma glucose after nasal administration of insulin. In the case of insulin solution without SG, the plasma glucose profiles are similar to that following PBS application without insulin. The glucose profiles

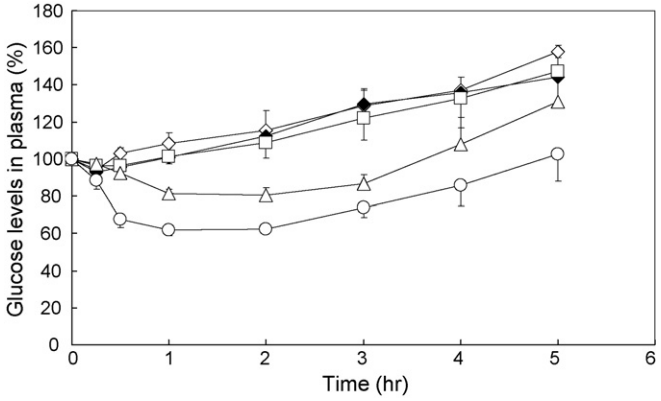

Fig. 1. Effect of insulin coadministered with different doses of SG on the glucose levels in plasma after nasal administration in rats. Control (PBS), ◇; insulin (10 IU/kg) alone, ♦; insulin (10 IU/kg) with SG 0.03%, □; insulin (10 IU/kg) with SG 0.1%, △; insulin (10 IU/kg) with SG 0.2%, ○. Each data set is the mean ± S.E.M. (n = 3–7).

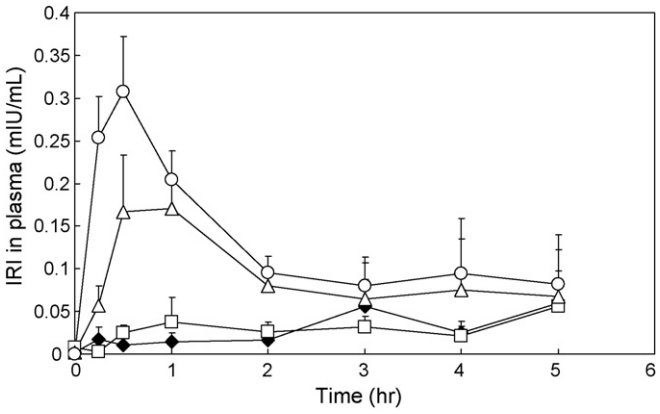

Fig. 2. Effect of different doses of SG on the IRI levels in plasma after nasal administration of insulin in rats. Insulin (10 IU/kg) alone, ♦; insulin (10 IU/kg) with SG 0.03%, □; insulin (10 IU/kg) with SG 0.1%, △; insulin (10 IU/kg) with SG 0.2%, ◯. Each data set is the mean ± S.E.M. (n = 3–7).

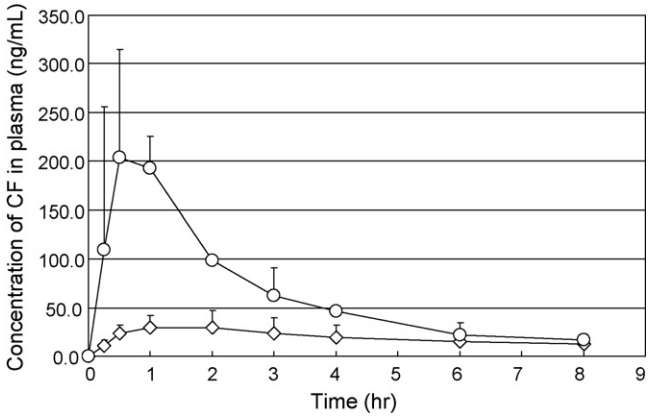

Fig. 3. Effect of SG (0.2%) on CF concentrations in plasma after nasal administration of CF (0.4 mg/kg) in rats. CF alone, ◇; CF with SG, ◯. Each data set is the mean ± S.E.M. (n = 3–5).

showing a continuous increase might be due to surgical damage and the effect of the anesthesia on the rats. In the cases of SG treatments, the plasma glucose levels fell to a degree that depended on the SG concentration. The maximum reduction (the lowest glucose concentration of 61.7% relative to baseline) was observed at 1 h in the rats treated with 0.2% SG.

The absorption of insulin was also detected by measuring IRI in plasma. Fig. 2 shows the plasma IRI levels after nasal administration of insulin with SG. The IRI levels increased to a degree that depended on the SG concentration. The area under the plasma IRI-time curve from 0 to 5 h (AUC_{IRI}) is shown in Table 2. The AUC_{IRI} value after 0.2% SG treatment was significantly higher than that after insulin administration without SG.

3.3. Nasal absorption of CF

In order to obtain information about the absorption-enhancing mechanism of SG, its effect on the nasal absorption of CF, as a paracellular marker, was examined. Fig. 3 shows the CF concentration in plasma after nasal administration of CF, with or without SG. The area under the plasma CF concentration-time curve from 0 to 8 h (AUC_{CF}) is shown in Table 3. The AUC_{CF} value with 0.2% SG was significantly higher than that without SG, suggesting an increase in the paracellular transport of CF.

3.4. Effect of SG on the Calu-3 cell monolayers

CF, FD4 and insulin were simultaneously applied to the apical side of the Calu-3 monolayers to determine their *Papp* in each monolayer. The *Papp* values are shown in Table 4. The penetration-enhancing effects of SG were evaluated in terms of the change in *Papp* for each penetrant. SG showed a significant enhancing effect for all penetrants. The enhancing ratios (*Papp* with SG/*Papp* without SG) were 1.8, 2.2 and 2.0 for CF, FD4 and insulin, respectively. The TER of the monolayers was reduced by addition of SG, suggesting that SG opened the intercellular tight junctions of the monolayers.

The membrane parameters of each Calu-3 cell monolayer were calculated from $Papp_{CF}$, $Papp_{FD4}$ and the previously reported diffusion parameters of CF and FD4 (Table 1) based on the Renkin function. Table 5 shows the R and ε/L values of the monolayers. The R values, equivalent pore radius of the permeation pathways, were about 10 nm in the treatments with and without SG. On the other hand, ε/L was increased significantly (about two-fold) following SG treatment. The ε/L appeared to be proportional to the area occupancy of the permeation pathways of CF and FD4 in the monolayers.

Table 3
The area under the plasma CF concentration–time curve from 0 to 8 h (AUC_{CF}) after nasal administration of CF and SG

	Control (without SG)	0.2% SG
AUC_{CF} (μg min/mL)	9.52 (3.05)[b]	32.3[a] (1.5)[b]
Ratio to control	–	3.4

[a] $P < 0.001$ in the t-test.
[b] S.E.M.

Table 2
The area under the plasma IRI–time curve from 0 to 5 h (AUC_{IRI}) after nasal administration of insulin and SG

	Control (without SG)	0.03% SG	0.1% SG	0.2% SG
AUC_{IRI} (mIU min/mL)	7.18 (3.68)[b]	8.77 (3.66)[b]	27.5 (9.9)[b]	37.9[a] (8.8)[b]
Ratio to control	–	1.2	3.8	5.3

[a] $P < 0.05$ in the Dunnett test.
[b] S.E.M.

Table 4
Effects of SG on the *Papp* of CF, FD4 and insulin, and TER of Calu-3 cell monolayers

	$Papp_{CF}$ (cm/s)	$Papp_{FD4}$ (cm/s)	$Papp_{insulin}$ (cm/s)	TER[a] (%)
Control (without SG)	3.57×10^{-7} (0.57×10^{-7})[b]	9.94×10^{-8} (2.51×10^{-8})[b]	1.28×10^{-9} (0.53×10^{-9})[b]	74.5 (10.8)[b]
SG 0.2%	6.26×10^{-7}[c] (0.70×10^{-7})[b]	2.15×10^{-7}[c] (0.25×10^{-7})[b]	2.53×10^{-9}[c] (0.24×10^{-9})[b]	12.2[d] (0.6)[b]

[a] The percentage of TER of the Calu-3 monolayers after the transport experiments compared with the initial values.
[b] SEM.
[c] $P < 0.05$ in the *t*-test.
[d] $P < 0.001$ in the *t*-test.

4. Discussion

In our previous study, ethylenediaminated gelatins (EG) having different numbers of amino groups were prepared and the absorption-enhancing effects on the nasal absorption of insulin and CF were examined in rats (Seki et al., 2005). The amino group content of EG was 0.40–0.81 mmol/g and the absorption-enhancing effect on insulin and CF depends on the amino group content. The amino group contents of the SG used in this study were 0.660 mmol/g for primary amino groups and 1.47 mmol/g for total amino groups. Since EGs have no additional secondary amino groups, comparison of EGs and SG with regard to the enhancing effects gives some information about the contribution of the secondary amino groups to these effects. In order to quantitate the absorption-enhancing effect on insulin, we calculated the *D*% value defined by the following equation:

$$D\% = \frac{AUC_{G,PBS} - AUC_{G,Insulin}}{AUC_{G,PBS}} \times 100 \quad (6)$$

where $AUC_{G,PBS}$ and $AUC_{G,Insulin}$ are the area under the curves of the plasma glucose levels from 0 to 5 h after nasal administration of PBS and insulin solution, respectively. The *D*% values after coadministration of 0.2% native gelatin (PA, 0.27 mmol/g), 0.2% EG (PA, 0.81 mmol/g) and SG (PA, 0.66 mmol/g; TA, 1.47 mmol/g) with 10 IU/kg insulin were 5.73 ± 3.20 (±S.E.M.), 25.7 ± 2.0 and 38.3 ± 3.7, respectively. In the addition, AUC_{CF} after nasal administration of CF with EG and SG were 18.4 ± 1.0 (μg min/mL) and 32.3 ± 1.5 (μg min/mL), respectively. These results suggest that the absorption-enhancing effects of the cationized gelatins are not only related to the primary amino group but also to the secondary one (Seki et al., 2006). Since the *D*% values were proportional to the PA of native gelatin and the EG and TA of SG according to the equation of $D\% = 27.2 \times$ PA or TA ($r^2 = 0.965$, $P < 0.005$), the primary and secondary amino group might be equivalent as far as in the enhancing effect is concerned.

Facilitated intercellular and intracellular permeability and inhibition of degradation during the permeation process are possible mechanisms of absorption-enhancement for peptide and protein drugs. The absorption experiments using CF as a paracellular marker were carried out to obtain some information about the enhancing mechanism of SG. When the enhancing ratios (ER) were expressed as the AUC ratios, the ER for insulin and CF in the 0.2% SG treatments were nearly equivalent, suggesting that facilitated intracellular permeability was the major mechanism whereby SG controlled the nasal absorption of insulin (Tables 2 and 3).

The Calu-3 cell monolayers were used in the *in vitro* experiments to investigate the enhancing mechanism of SG more clearly. Although Calu-3 is a human bronchial epithelial cell line, it can be used as a model airway epithelial cell to evaluate nasal absorption of drugs (Claudia and Randall, 1999; Nagendry et al., 2005). Most of the candidate enhancers reported are able to open the tight junctions of epithelial cell layers and increase the paracellular permeability of drugs. There are two different ways to open the tight junctions (Seki et al., 2006). If the enhancers expand the size of each permeation pathway for peptide and protein drugs in the tight junctions, larger molecules will be able to pass via these routes. If the enhancers do not expand the size but increase the number of the pathways by transformation from true tight junctions into loose ones, the enhancing effect for each drug is independent of their size. In this study, the Renkin molecular sieving function was applied to identify the mode of opening of the tight junctions. The results showed that ε/L increased about two-fold, while *R* was not changed by the SG treatment (Table 5). This means that SG transforms the

Table 5
Effects of SG on the permeation characteristics of the paracellular pathway of Calu-3 cell monolayers based on the Renkin molecular sieving function

	ε/L (cm^{-1})	*R* (nm)	Calculated $P_{insulin}$[a] (cm/s)	*P* ratio (observed/calculated)
Control (without SG)	0.0775 (0.0083)[b]	10.4 (2.4)[b]	2.17×10^{-8} (1.00×10^{-8})[b]	0.0586 (0.0163)[b]
SG 0.2%	0.157[c] (0.017)[b]	11.7 (1.2)[b]	4.96×10^{-8} (0.95×10^{-8})[b]	0.0554 (0.0064)[b]

[a] Calculated permeability of insulin from the diffusion coefficient and Stokes–Einstein radius of insulin (Table 1) based on the Renkin function.
[b] S.E.M.
[c] $P < 0.05$ in the *t*-test.

true tight junctions, which function as a perfect barrier for water-soluble molecules, into pathways of CF and FD4 to increase the number. The Renkin function can also be used for the calculation of the *P*-value of a drug with a known D_i and r_i. The calculated *P*-values of insulin, with and without SG, through the Calu-3 monolayers are shown in Table 5. The calculated *P*-values were higher than the corresponding *Papp* values of insulin obtained in the permeation experiments and the ratio (observed/calculated) was about 0.06. This low value indicates degradation of insulin during the permeation process through the Calu-3 monolayers and similar values with and without SG indicates that SG does not inhibit the degradation of insulin. The enhancing mechanism of SG for insulin permeation involves an increase in the number of permeation pathways in the tight junctions, at least in the Calu-3 cell monolayers. The use of protease inhibitors together with SG could be one way of obtaining a higher bioavailability of insulin also after nasal application (Quan et al., 1999).

In summary, SG exhibited enhancing effects on the nasal absorption of insulin and CF in rats and the permeation of CF, FD4 and insulin through Calu-3 cell monolayers. SG was able to increase the number rather than the size of the pathways for water-soluble molecules in the Calu-3 cell monolayers. SG is a good candidate as a safe absorption enhancer to produce a minor modification of the permeability of the paracellular pathway of mucosal membranes, while retaining the sieving property of the epithelial membranes.

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