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Research paper

Improvement of intestinal absorption of peptides: adsorption of B1-Phe monoglucosylated insulin to rat intestinal brush-border membrane vesicles

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Abstract

In a previous study we glycosylated insulin to improve its intestinal absorption. When the glycosylated product, *p*-(succinylamido)-phenyl-α-D-glucopyranoside (SAPG)-substituted insulin (SAPG-INS), was administered intra-intestinally to rats, it showed a greater hypoglycemic effect than native bovine insulin. The enhanced hypoglycemic effect of SAPG-INS was considered to be due to an increase in membrane permeability as well as an increase in resistance to enzymatic degradation. In particular, membrane permeability may be related to an interaction with the Na^+-dependent D-glucose transporter (SGLT-1) which is located in the brush-border membrane of epithelial cells. The insulin product used in the previous study, however, comprised a mixture of mono-, di- and tri-SAPG-substituted insulin. In this study SAPG-INS with a defined substitution number and position was synthesized to examine the interaction between the transporter and glycosylated insulin in more detail. The new product was mono-SAPG-substituted insulin substituted at the B1-phenylalanine position (B1-SAPG-INS) and was selectively synthesized after protection of the A1-glycine and εB29-lysine amino acids. The hypoglycemic effect of B1-SAPG-INS in rats after an intravenous dose of 71 μg/kg was almost the same as that of native bovine insulin at a dose of 1 U/kg and B1-SAPG-INS retained about 60% of the immunoreactivity of native bovine insulin. The interaction of B1-SAPG-INS with the intestinal transporter was examined by a rapid filtration technique using ^{125}I-labeled B1-SAPG-INS and brush-border membrane vesicles (BBMVs) which were prepared from rat small intestine by the Mg-precipitation method. The amount of B1-SAPG-INS adsorbed or absorbed by BBMVs in the presence of an inward Na^+-gradient into BBMVs was greater than that of native bovine insulin. This adsorption/absorption was significantly inhibited by the presence of 1 mM phloridzin. A similar inhibition was observed when Na^+ was replaced with K^+ and when B1-SAPG-INS was incubated with BBMVs at 4°C. From the effect of osmolarity on the extent of adsorption/absorption, it was considered that B1-SAPG-INS was not taken up into the intravesicular space but adsorbed onto the external membrane surface of BBMVs. These findings suggested that B1-SAPG-INS was adsorbed specifically onto the transporter. The hypoglycemic effect of insulin was enhanced by glycosylation at the B1 position in in situ experiments using normal and diabetic rats. Consequently, it is suggested that B1-SAPG-INS was adsorbed specifically onto the glucose transporter of intestinal BBM. This specific adsorption may be involved in the mechanism of the enhanced hypoglycemic effect of B1-SAPG-INS both in normal and diabetic rats.

Keywords: Glycosylation; Insulin; Na^+-dependent D-glucose transporter; Hypoglycemic effect; Brush-border membrane vesicles

1. Introduction

The route of administration of insulin is still limited to injection although many studies have been carried out in order to develop non-parenteral routes. The two main barriers to oral administration of insulin are; (1) inactivation by digestion in the gastrointestinal tract and, (2) low enterocyte permeability.

Previously, we modified insulin (INS) with *p*-succinylamidophenyl (SA)-α-D-glucopyranoside (SAPG), SA-α-D-mannopyranoside (SAPM) and SA-α-L-arabinopyranoside (SAPA) to enhance its intestinal permeability and studied the hypoglycemic effects of the modified factors in rats [1]. It was shown that the hypoglycemic effect of SAPG-INS was greater than that of SAPM-INS and that SAPA-INS was ineffective. It was suggested that the increased resistance of insulin to enzymatic degradation as a result of

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glycosylation could contribute to its hypoglycemic effect. In addition, it was also suggested that glucose attached to insulin could interact with the Na^+-dependent D-glucose transporter (SGLT-1) on the intestinal wall resulting in an increased number of insulin molecules in close proximity to the wall leading to an enhancement of its intestinal permeability. To increase the resistance to enzymatic degradation, co-administration of an enzyme inhibitor [2–4] and the use of various carriers [5,6] have been studied. The use of absorption enhancers [7], chemical modification by lipids [8] and saccharides [1] have been also studied in order to increase intestinal absorption. The SAPG-INS used in our previous study was a mixture of A1-Gly or B1-Phe monosubstituted INS, A1-Gly, B1-Phe di-substituted INS and A1-Gly, B1-Phe, εB29-Lys tri-substituted INS. Seminoff et al. [9] reported that the bioactivity of B1-Phe monosubstituted SAPG-INS (B1-SAPG-INS) was the highest and was comparable to that of native insulin, and that of A1-Gly monosubstituted SAPG-INS showed a slight decrease in its maximal effect. The A1,B1-disubstituted SAPG-INS showed significantly reduced bioactivity (about 60%) and the A1,B1,B29-trisubstituted SAPG-INS had no bioactivity. Baudys et al. [10,11] synthesized seven SAPG-insulin derivatives and studied their biological activity and physical stability. They reported that the most pronounced site-specific modification was that occurring on the B1-Phe site of insulin.

In this study we selectively synthesized B1-SAPG-INS from A1, εB29 di-protected INS to examine the interaction between the transporter and glycosylated INS in more detail using brush-border membrane vesicles (BBMVs) which were prepared from rat small intestine. We then studied the hypoglycemic effect of B1-SAPG-INS using the in situ Doluisio method.

2. Materials and methods

2.1. Materials

Lactoperoxidase, bovine insulin, dansylglycine, dansyl-L-phenylalanine, and *N*ε-dansyl-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO). Dimetylphosphinothioyl chloride (Mpt-Cl) was purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan). *p*-Nitrophenyl-α-D-glucopyranoside (α-NPG), palladium carbon and dansyl chloride were purchased from Nakalai Tesque Inc. (Kyoto, Japan). 4-(*p*-Methoxybenzyloxycarbonyloxy)phenyldimethyl sulfonium methylsulfate (pMZ-DSP) was obtained from Sanshin Chemical Ind. Co., Ltd. (Yamaguchi, Japan). $Na^{125}I$ was purchased from Amersham Japan (Tokyo, Japan). Insulin RIA beads II kit and Nembutal® Injection were obtained from Dainabot Co., Ltd. (Tokyo, Japan). Humulin® R 100 was purchased from Eli Lilly Japan K.K. (Kobe, Japan). Con A-agarose was purchased from Seikagaku Corp. (Tokyo, Japan). DEAE Sephadex A-25 and PD-10 were from Pharmacia Biotech (Uppsala, Sweden). Centriprep-3 was purchased from Grace Japan K.K., Amicon (Tokyo, Japan). The Bio-Rad protein assay kit and the Glucose B-Test Wako and Alkaline phospha K-Test Wako kits were obtained from Bio-Rad Lab. (Richmond, CA) and Wako Pure Chemical Ind. Ltd. (Osaka, Japan), respectively. All other chemicals were analytical grade and were used without further purification. Deionized redistilled water was used to prepare all solutions.

2.2. Synthesis and purification

2.2.1. Synthesis of SAPG

The synthesis of SAPG was performed according to the method of Jeong et al. [12].

Briefly, the nitrophenyl group on α-NPG was reduced to an amionophenyl group with ammonium formate in the presence of palladium carbon yielding *p*-aminophenyl-α-D-glucopyranoside (α-APG). APG was then allowed to react with succinic anhydride at room temperature for 4 h to produce SAPG.

2.2.2. Synthesis of A1-Gly and εB29-Lys-protected INS by pMZ (A1, εB29-pMZ-INS)

pMZ-protected INS (pMZ-INS) was synthesized according to the method of Kouge et al. [13] with some modifications. Bovine-INS (90 mg, 15.7 μmol) was dissolved in a 1:1 (v/v) mixture of water and dimethylformamide (DMF, 30 ml total volume) and the pH of the solution was adjusted to 10 with 0.1 N NaOH. The solution was stirred for 2.5 h at room temperature while adjusting the pH from 9.90 to 10.1 with 0.1 N NaOH. Then 20% citric acid was added to adjust the pH to 3.0. The solution was dialyzed using a Spectra/Por 3 dialysis membrane (Spectrum Medical Industries, Inc., Los Angeles, CA; molecular cut-off, 3500) at 4°C first in water then in 0.01 M Tris–HCl buffer solution (pH 7.9) containing 60 mM NaCl and 7 M urea. pMZ-INS was then applied to a DEAE Sephadex A-25 column (2.5 × 50 cm) and eluted with the above 0.01 M Tris–HCl buffer with a NaCl concentration gradient expressed as follows:

$$C = C_a + (C_0 - C_a)\exp(-K_0 t/V)$$

where $C_a = 220$ mM, $C_0 = 60$ mM, $K_0 = 40$ ml/h, $V = 1500$ ml; t, time (h).

The UV absorption of each fraction (17 ml) was measured at 278 nm using a Hitachi U-3000 recording spectrophotometer and the fractions showing increased absorption were collected, pooled and dialyzed against water followed by lyophilization.

2.2.3. Selective synthesis of SAPG-INS

The coupling of SAPG to A1, εB29-pMZ-INS was performed using the Mpt (dimethyphosphinothioyl) mixed anhydride method according to Ueki et al. [14]. Briefly, after 120 mg SAPG (320 μmol) was dissolved in 1.08 ml DMF containing 80 μl (320 μmol) tri-*n*-butylamine, 34 μl

(320 μmol) Mpt-Cl was added at 0°C and the solution was stirred for 15 min. Next, 160 μl (640 μmol) tri-n-butylamine was added. A1,B29-pMZ-INS (100 mg, 16.5 μmol) was dissolved in a 1:1 (v/v) mixture of water and DMF (total volume 36 ml) which was cooled in an ice bath and the pH adjusted to 9.5 with 0.1 N NaOH. The SAPG solution was added dropwise to this A1,B29-pMZ-INS solution in an ice bath and the pH of the mixed solution was adjusted to 9.5 with 0.1 N NaOH. After the solution was allowed to stand at room temperature for 1 h, it was stirred overnight while maintaining the pH at 9.5. It was then dialyzed in water at 4°C and lyophilized. SAPG-A1,B29-pMZ-INS (20 mg) was dissolved in a 1:4 (v/v) mixture of anisol and trifluoroacetic acid (TFA, total 2.5 ml) which was cooled in an ice bath and the solution stirred. After 60 min, TFA was removed by evaporation at 30°C and the crude SAPG-INS was triturated by treating the residue with ethylether. The crude SAPG-INS was washed with ethylether and separated by centrifugation (3000 rev./min for 10 min). This procedure was repeated several times until no odor of anisol was detected. After the ethylether was removed, the crude SAPG-INS was dissolved in 30 ml Con A buffer solution (20 mM Tris–HCl containing 500 mM NaCl, 1 mM $CaCl_2$ and 1mM $MnCl_2$ (pH 7.4)) and concentrated to 6–12 ml using a Centriprep-3 (MW cut-off; 3000).

2.2.4. Purification of B1-Phe monoglycosylated SAPG-INS (B1-SAPG-INS)

The crude SAPG-INS (10 mg in 3–6 ml Con A buffer solution) was applied to a Con A-agarose gel column (1 × 10 cm) which had been equilibrated with 100 ml Con A buffer solution. After an adsorption time of 40 min, the buffer solution was eluted at a flow rate of 10 ml/h. The UV absorption of each fraction (2.5 ml) was measured at 276 nm. After confirming that there was no increased absorption, 20 mM Tris–HCl buffer solution containing 100 mM methyl-α-D-mannoside (pH 7.4) was eluted and the fractions showing increased absorption at 276 nm were collected and pooled. The solution was dialyzed against water and lyophilized to give purified B1-SAPG-INS.

2.3. Determination of B1-SAPG-INS

2.3.1. Determination of sugar groups and HPLC analysis

The number of sugar groups on B1-SAPG-INS was determined using the phenol/sulfuric acid method [15] and a Micro BCA protein assay reagent kit (Pierce, Rockford, IL) as described previously [16]. From the observed concentrations of sugar and peptide, the sugar/peptide ratio of B1-SAPG-INS was shown to be 1. HPLC analysis of synthesized pMZ-INS and B1-SAPG-INS was performed using a Waters 600E system (484 UV/Vis detector, 741 Data Module, Millipore Corp., Waters Chromatography Division, Milford, MA) on a Protein Pak G-QA column (8.2 × 75 mm, Millipore Corp., Waters Chromatography Division, Milford, MA) with a Tris–HCl concentration gradient. Eluants A and B comprised 5 and 250 mM Tris–HCl buffer solution containing 4 M urea (pH 8.5), respectively. The ratio of A:B was 93:7 (v/v, initial) was increased linearly to 0:100 over 240 min. The mobile phase flowed at a rate of 1.0 ml/min and the absorbance at 280 nm was measured.

2.3.2. Analysis of the position of SAPG substitution

The position of pMZ and SAPG-substitution on INS was estimated according to the method of Gray [17] with some modifications. Briefly, a sample (0.1–0.8 mg) was placed in a dried small test tube and 50 μl of a 1% SDS solution was added. The protein was dissolved by heating in a boiling water bath for 5 min. After cooling, 50 μl *N*-ethylmorpholine was added, mixed and then 75 μl dansyl chloride (DNS-Cl) was added. This solution was allowed to react for 24 h at room temperature. After solidification by passing N_2 gas over the sample, 50 μl water was then added and the protein was precipitated by the addition of 1 ml acetone. The precipitate was collected as a pellet by centrifugation (3000 rev./min) for 5 min then it was washed with acetone and centrifuged again. This procedure was repeated three times. After the evaporation of acetone by N_2 gas, the precipitate was dried under a vacuum. Then, 100 μl 6 N HCl was added and the glass tube was sealed. Hydrolysis was carried out at 105°C for 36 h. The glass tube was unsealed, centrifuged (3000 rev./min) then HCl was removed under a vacuum. The DNS-amino acids produced were analyzed by two-dimensional TLC using Polyamide 11 F_{254} (Nacalai Tesque, Inc.). Developing solvent I consisted of ethylacetate/chloroform/methanol/acetic acid (10:10:1:1 v/v) and solvent II consisted of benzene/acetic acid (4:1 v/v). Amino acid analysis was performed by Toray Research Center Inc. (Tokyo, Japan).

2.3.3. Immunoreactivity of B1-SAPG-INS

The protein assay of B1-SAPG-INS was made using a Bio-Rad protein assay kit. The calibration curve was constructed using bovine crystalline insulin (0–25 μg/ml) which had been dissolved in a 9:1 (v/v) mixture of 50 mM glycine–HCl buffer solution (pH 2.6) and Con A buffer solution. The purified B1-SAPG-INS was dissolved in the same buffer solution and diluted to approximately 10–20 μg/ml. The protein concentration of this solution was then determined using the calibration curve. The immunoreactivity of this solution was determined using an Insulin RIA beads II kit. The calibration curve was made using the standard insulin solution (0–300 μU/ml) included in the kit. Radioactivity was measured in an auto-well gamma counter (ARC-300, Aloka Co. Ltd., Tokyo, Japan). From these values, the immunoreactivity of B1-SAPG-INS was obtained as 15 IU/mg protein.

2.4. In vivo experiment

All procedures using animals were conducted in accordance with the NIH Guide for the Care and Use of Lab-

oratory Animals and were approved by the animal ethics committees of the Faculty of Pharmaceutical Sciences, Science University of Tokyo. Male Wistar rats (body weight 200 ± 20 g, obtained from Nippon Biomaterials Center, Tokyo, Japan) were fasted for 18–24 h prior to anesthetization with pentobarbital sodium (50 mg/kg) which was given intrapertioneally then rats were fixed on their backs on a board. The right jugular vein was cannulated with heparinized polyethylene tubing and B1-SAPG-INS (5 IU/kg) was injected from the right femoral vein. At set time intervals after injection, blood samples (100 μl) were withdrawn from the jugular vein cannula and collected in heparinized tubes. After centrifugation (3500 rev./min, 5 min) 20 μl plasma was taken from the blood samples and the blood glucose level was assayed using a Glucose B-Test Wako. In some experiments, rats were made diabetic by the intraperitoneal injection of streptozotocin (STZ, 60 mg/kg).

2.5. In vitro and in situ experiments

2.5.1. Preparation of brush border membrane vesicles (BBMVs)

BBMVs were prepared by Mg^{2+} precipitation according to the methods of Tiruppathi et al. [18] and Takuwa et al. [19] as described previously [16].

2.5.2. Iodination of B1-SAPG-INS

B1-SAPG-INS was iodinated according to the method of Hamlin et al. [20] with some modifications. Briefly, 2.5 mg B1-SAPG-INS was dissolved in 625 μl 0.01 N HCl then 625 μl 0.1 M sodium citrate buffer (pH 5.2) and 10 μl 125 mM EDTA disodium solution were added. To this solution, 2 μl 0.05M H_2O_2, 2 μl KI/$Na^{125}I$ mixture solution (0.05 M KI; 3.7 GBq/ml $Na^{125}I$; 3:1 by v/v) and 2 μl lactoperoxidase solution in 125 mM EDTA (final concentration of lactoperoxidase 9.25 mM) were added successively while stirring at time points of 0, 10 and 20 min, respectively. Ten minutes after the last addition, the mixture was centrifuged at 12 000 × *g* for 15 min and the precipitate was washed twice in 1 ml 50 mM ammonium acetate buffer (pH 5.2). The resultant pellet was dissolved in uptake buffer (16 mM Tris–10 mM HEPES–HCl (pH 7.5) containing 15 mM KCl and 270 mM mannitol) and then fractionated into labeled B1-SAPG-INS and other fractions using a PD-10 column (Sephadex G-25; 1.6 × 5.2 cm). The radioactivity of each 0.6 ml fraction was determined in an auto-well gamma counter (Aloka, ARC-300) and protein concentration was determined using a Bio-Rad protein assay kit.

2.5.3. Adsorption or uptake of B1-SAPG-INS to BBMVs

Adsorption or uptake of B1-SAPG-INS to BBMVs was measured at 4 or 37°C by a rapid filtration technique according to McCarthy et al. [21]. The ^{125}I-labeled B1-SAPG-INS solution eluted from the PD-10 column was diluted 10 times with 16 mM Tris–10 mM HEPES–HCl containing 15 mM KCl and 144.4 mM NaCl (or 166.1 mM KCl without NaCl). This labeled B1-SAPG-INS solution and BBMVs suspension were incubated separately at 4 or 37°C. After 5 min, 300 μl of the BBMVs suspension was added to 1 ml labeled B1-SAPG-INS solution, mixed then incubated at 4 or 37°C. The osmolarity of this mixed solution was 571 mOsm/l, which was measured using a osmometer (Osmette, Precision Systems Inc.). Hyperosmolar buffer solutions (668, 750 and 837 mOsm/l) were prepared by adjusting the amount of D-mannitol added to each solution. At set time intervals after mixing, the suspension (100 μl) was sampled from a plastic test tube and sucked through a prewashed nitrocellulose filter with a pore size of 0.45 μm (Advantec Toyo, Tokyo, Japan). The filter was washed three times with 4 ml ice-cold stop solution (10 mM Tris–11 mM HEPES–HCl (pH 7.5) containing 153 mM NaCl). The filter was transferred to an RIA tube and radioactivity was measured in an auto-well gamma counter. The blank value was determined by mixing the labeled B1-SAPG-INS solution with the vesicle suspension followed by immediate filtration and washing with stop solution. This value was subtracted from the uptake data.

2.5.4. Intestinal absorption of B1-SAPG-INS (in situ Doluisio method)

Male Wistar rats were treated as described in 'in vivo experiment'. The right jugular vein was cannulated with a heparinized polyethylene tube. After making a median incision in the abdomen, the small intestine was pulled out and cut at two positions, i.e. 2 and 12 cm from Treiz's ligaments. Two L-type glass cannulae (inner and outer diameter, 3 and 5 mm, respectively) were inserted into the ends of these positions and fixed into position with a ligature. This jejunal segment (10 cm) was rinsed with physiological saline solution. The glass cannulae were connected by silicon tube to 10 ml disposable syringes which had a small air hole in the middle of their barrels. The abdomen was closed and the skin sutured. B1-SAPG-INS (50 IU/kg, 10 IU/6 ml ammonium acetate buffer (pH 6.9)) was administered into the intestinal segment from the one syringe and the solution, which was transferred to the other syringe, was flowed back again. This mixing procedure was repeated three times and the solution level in both syringes was adjusted so that it was equal. Every 30 min after administration, blood samples (100 μl) were withdrawn from the jugular vein cannula and collected in heparinized tubes. After centrifugation (3500 rev./min, 5 min) 20 μl plasma was sampled and the blood glucose level was assayed using a Glucose B-Test Wako. The mixing procedure was repeated after each blood sample was taken.

3. Results

3.1. Selective synthesis of B1-SAPG-INS

The elution pattern of pMZ-INS by DEAE Sephadex A-

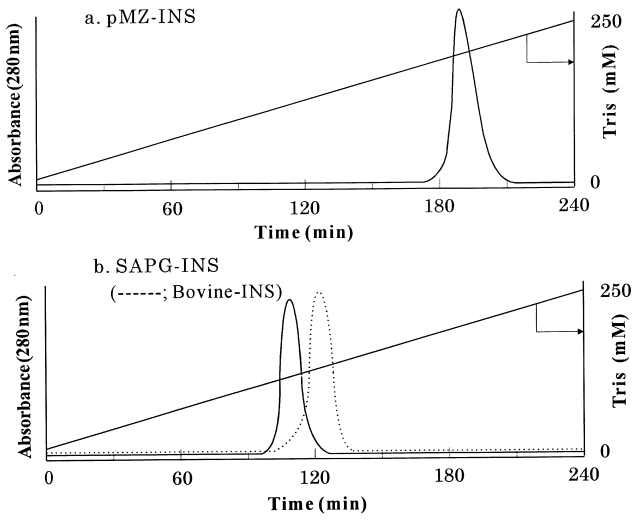

Fig. 1. Ion-exchange HPLC chromatograms of pMZ-INS (a) and SAPG-INS (b). The concentration of Tris–HCl was increased linearly from 22 to 250 mM over 240 min.

25 showed one small peak at about 120 mM NaCl gradient and one main peak at about 160 mM NaCl gradient (data not shown). The retention time of the dialyzed and lyophilized product of the main peak fractions was 200 min when it was analyzed by ion exchange HPLC (Fig. 1a) while the retention time of bovine-INS was about 125 min (Fig. 1b). The two-dimensional TLC profile of dansylated hydrolysates of the lyophilized product of the main peak is shown in Fig. 2c. Only one spot for DNS-L-Phe was observed. As the pMZ-protected terminal groups of INS were not dansylated, the lyophilized product of the main peak was identified as A1-Gly, εB-29-Lys- diprotected INS (A1, εB29-pMZ-INS).

After the coupling of SAPG to A1, εB29-pMZ-INS using an Mpt mixed anhydride, SAPG-pMZ-INS was treated with TFA and anisol then the crude SAPG-INS was purified on a Con A column. Only one product (product I) was obtained and had a retention time at 116 min (Fig. 1b). Fig. 2d shows the two-dimensional TLC profile of dansylated hydrolysates of product I. Two spots corresponding to DNS-L-Gly and Nε-DNS-Lys were obtained. From this finding, product I was identified as B1-SAPG-INS. The amino acid composition of B1-SAPG-INS was further analyzed by the ninhydrin method to confirm the integrity of insulin. As the relative numbers of each amino acid of B1-SAPG-INS (expressed in values relative to the number of Leu, where Leu = 6) were virtually identical to those for bovine insulin (data not shown), the composition of insulin of B1-SAPG-INS was shown to be intact.

The bioactivity of synthesized B1-SAPG-INS was then tested by i.v. administration of B1-SAPG-INS to rats. The immunoreactivity of B1-SAPG-INS was 15 IU/mg, as already described in Section 2.3.3, which was about 40% lower than that for intact bovine insulin. Fig. 3 shows the changes in blood glucose level (BLG) after i.v. administration of B1-SAPG-INS and bovine insulin at a dose of 1 IU/kg. B1-SAPG-INS (71 μg/kg) showed almost the same hypoglycemic effect as that of bovine insulin (40 μg/kg) and the BGL returned to its original level after 360 min. This suggested that the immunoreactivity of B1-SAPG-INS directly reflected its biological effect.

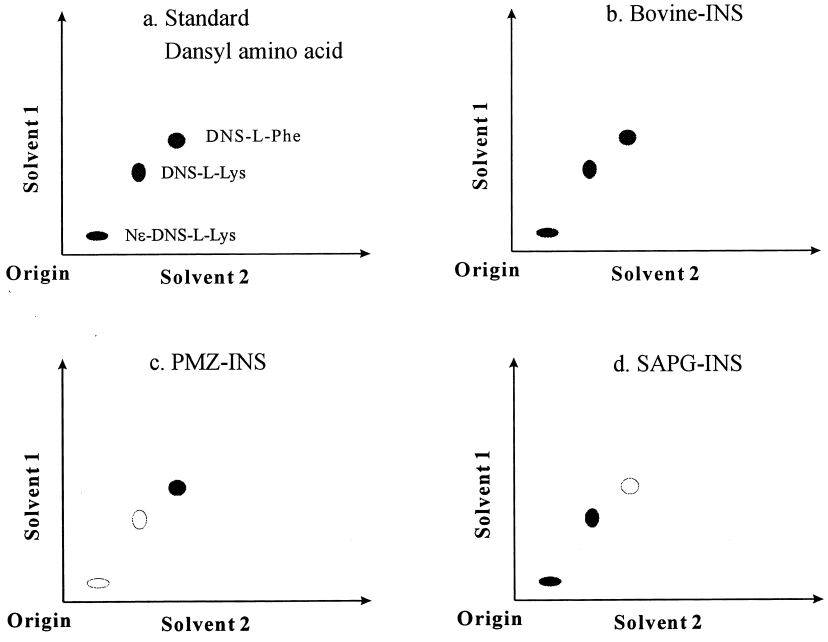

Fig. 2. Two-dimensional TLC profiles of dansylated hydrolysates of standard dansyl amino acids (a), bovine-INS (b), pMZ-INS (c) and SAPG-INS (d). The developing solvent I consisted of ethylacetate/chloroform/methanol/acetic acid (10: 10: 1: 1 v/v) and solvent II consisted of benzene/acetic acid (4: 1 v/v).

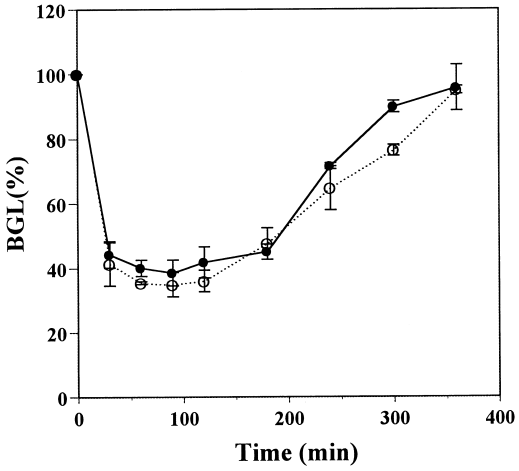

Fig. 3. Changes in blood glucose level (BGL) after intraveneous administration of B1-SAPG-INS in rats. The results are given as means of three experiments. Key: ○, B1-SAPG-INS (1 IU/kg); ●, bovine-INS (1 IU/kg).

3.2. Adsorption of B1-SAPG-INS to BBMVs

The interaction of B1-SAPG-INS with the SGLT-1 transporter was subsequently investigated using BBMVs. Time-course changes in adsorption/absorption of B1-SAPG-INS to BBMVs are shown in Fig. 4. Only weak adsorption (about 0.02 μg/mg BBM protein) was observed 20 min after the start of incubation when bovine insulin was incubated in the presence of 144 mM NaCl. In contrast, about 0.08 μg insulin/ mg BBM protein was adsorbed or absorbed 20 min after the start of incubation when B1-SAPG-INS was incubated in the presence of 144 mM NaCl and no overshoot uptake was observed. This adsorption/absorption of insulin was significantly inhibited both in the presence of 1 mM phloridzin and when Na^+ was replaced with K^+.

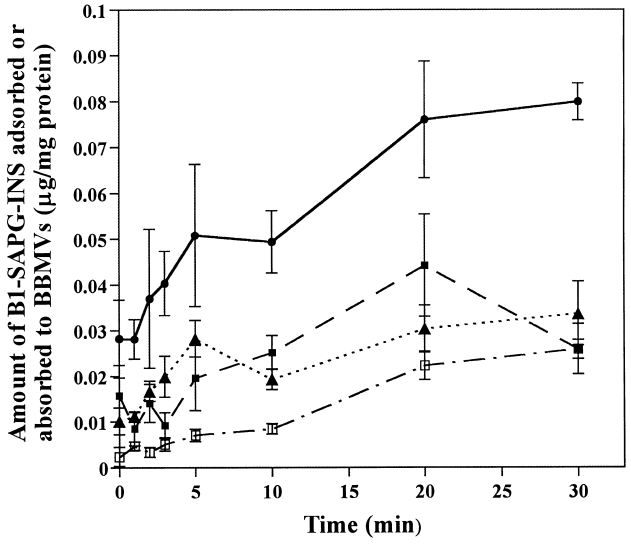

Fig. 4. Time-course changes of adsorption/absorption of B1-SAPG-INS to BBMVs. The results are given as means ± SE ($n = 4$). Key: □, bovine-INS; ●, SAPG-INS with Na^+ gradient; ▲, SAPG-INS with no Na^+ gradient; ■, SAPG-INS with phloridzin.

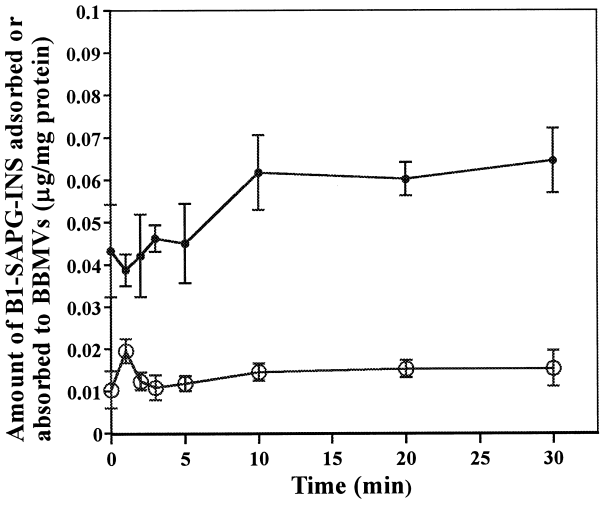

Fig. 5. Adsorption/absorption of B1-SAPG-INS to BBMVs at 4 and 37°C. The results are given as means ± SE ($n = 4$). Key: ●, 37°C; ○, 4°C.

When B1-SAPG-INS was incubated with BBMVs at 4°C, the adsorption/absorption was significantly inhibited (Fig. 5). As non-specific adsorption did not decrease at low temperature, the difference between the adsorbed or absorbed amounts at the two temperatures was considered to be due to specific binding of B1-SAPG-INS to SGLT-1. The extent of adsorption/absorption of B1-SAPG-INS was then analyzed by determining it as a function of the reciprocal of osmolarity (Fig. 6). In spite of the increase in osmolarity, the extent of adsorption/absorption of B1-SAPG-INS was almost constant indicating that B1-SAPG-INS was not taken up into the intravesicular space but was adsorbed onto the external membrane surface of BBMVs.

3.3. Hypoglycemic effect of B1-SAPG-INS

The hypoglycemic effect of B1-SAPG-INS was studied by

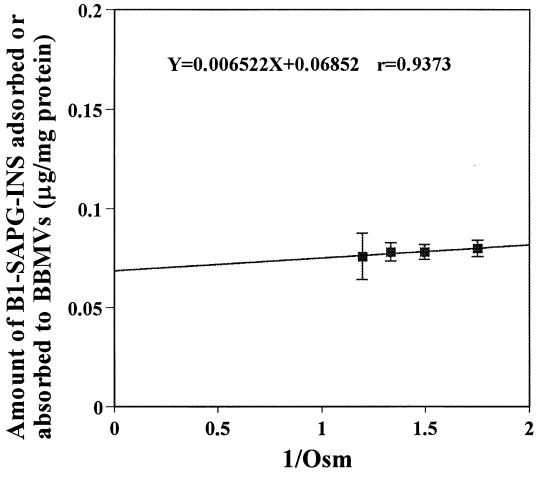

Fig. 6. Effect of osmolarity on the adsorption/absorption of B1-SAPG-INS to BBMVs. The results are given as means ± SE ($n = 4$).

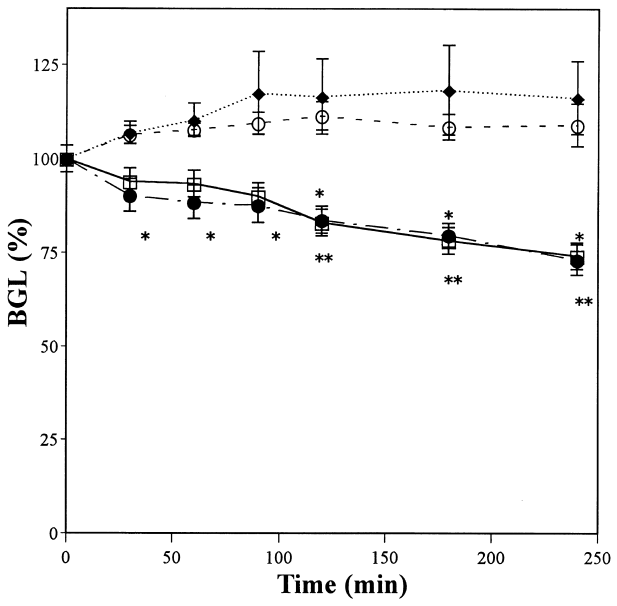

Fig. 7. Changes in blood glucose level (BGL) after administration of B1-SAPG-INS in normal and diabetic rats. The results are given as means ± SE ($n = 4$), *$P < 0.05$ vs. control (bovine-INS); **$P < 0.01$ vs.control (bovine-INS). Key: □, B1-SAPG-INS (50 IU/kg) in normal rats; ◆, control in normal rats (bovine-INS, 50 IU/kg); ●, B1-SAPG-INS (50 IU/kg) in diabetic rats; ○, control in diabetic rats (bovine-INS, 50 IU/kg).

the in situ Doluisio method using rats. Fig. 7 shows the changes in BGL after administration of B1-SAPG-INS in both normal and diabetic rats. The average BGL in normal rats before administration was about 130 mg/dl. When 50 IU/kg bovine-INS was administered, BGL increased by a maximum of 15% compared to the initial value during the 250-min period after administration. This slight increase in BGL may be mainly due to stress during the experiment. BGL decreased by a maximum of 25% compared to the initial value during the 250-min period when 50 IU/kg B1-SAPG-INS was administered and then it recovered to the initial value 360 min after administration (data not shown). These findings indicated that the hypoglycemic effect of INS was enhanced by glycosylation at the B1 position. The hypoglycemic effect of B1-SAPG-INS was then studied in STZ-induced diabetic rats. Changes in BGL after administration of B1-SAPG-INS in diabetic rats are also shown in Fig. 7. The average BGL of diabetic rats before administration was about 400 mg/ml. BGL increased by a maximum of 15% compared to the initial value during the 250-min period after administration in the control while BGL decreased by a maximum of 25% during the 250-min period after administered in a similar manner to that observed in normal rats. In the case of diabetic rats, the extent of the BGL decrease was larger than that seen in normal rats and the hypoglycemic effect lasted up to 360 min.

4. Discussion

In a previous paper [1] we studied the hypoglycemic effect of intestinally administered monosaccharide-modified insulin derivatives in rats and reported that the order of the hypoglycemic effect was SAPG-INS > SAPM-INS (*p*-succinylamidophenyl-α-D-mannopyranoside-modified insulin) > SAPA-INS (*p*-succinylamidophenyl-α-L-arabinopyranoside-modified insulin). It was reported [22] that the relative affinity of Na^+-dependent D-glucose transporter for monosaccharides was in the order of D-glucose > D-mannose > L-arabinose = 0. L-Arabinose is considered to have no affinity since it does not exist in nature. From these parallel relationships it was considered that the SGLT-1 transporter played an important role in the hypoglycemic effect of SAPG-INS. In this study we synthesized B1-SAPG-INS which had a defined substitution number and position of SAPG. We then examined the interaction between B1-SAPG-INS and BBMVs and its hypoglycemic effect.

Seminoff et al. [9] prepared B1-SAPG-INS or A1-Gly mono-substituted INS, A1-Gly, B1-Phe- di-substituted INS and A1-Gly, B1-Phe, εB29-Lys- tri-substituted INS from SAPG-INS mixtures by separation using FPLC (fast protein liquid chromatography). In such preparative liquid chromatography, however, the amount of product obtained in one separative step is generally limited and in our case many cycles were required to prepare a large amount of product. We therefore tried to selectively synthesize B1-SAPG-INS using a protecting group. Baudys et al. [10,11] also synthesized SAPG-insulin derivatives using a Boc protecting group. In a previous study we used isobutyl chloroformate (IBCF) to introduce SAPG into INS [1]. In this mixed anhydride method using IBCF, however, there were some disadvantages such as the tendency for disproportionation and a need for protection of the side-chain hydroxyl functions. We used dimethylphosphinothioyl chloride (Mpt-Cl) instead of IBCF in this series of experiments to overcome these disadvantages.

It is well known that phloridzin competes with α-D-glucosides for the SGLT-1 transporter so it might inhibit the binding of B1-SAPG-INS to the transporter. The reason for the decreased amount of B1-SAPG-INS adsorbed or absorbed in the absence of Na^+ was considered to be because Na^+ was necessary for the binding of α-D-glucosides to the transporter or because B1-SAPG-INS was actively transported into BBMVs. Hirayama et al. [23] studied the cationic effects on protein conformation and transport in the Na^+/glucose cotransporter and reported that substitution of H^+ or Li^+ for Na^+ caused a decrease in apparent affinity for sugar by one order of magnitude or more. They interpreted the results by assuming that binding of the cation caused a conformational change in the sugar binding pocket and that the exact conformation was determined by the specific cation.

In separate studies using tetrapeptide (α and β-SAPG-GGYR), we found that the glycosylated α and β-SAPG-GGYR were transported by SGLT-1 [16]. In addition, we have reported that the glycosylated pentapeptides, α and β-

SAPG-Leu-enkephalin (α, β-SAPG-LE) were also transported by this transporter but the amount of α, β-SAPG-LE transported was lower than that of α, β-SAPG-GGYR (M. Yamada et al., personal communication). We considered from these observations that the molecular size of the glycosylated peptide transported by SGLT-1 was limited and that the size of the pentapeptide may be near the upper limit. It is unlikely, therefore, that SAPG-INS is taken up into BBMVs by SGLT-1 from the viewpoint of molecular size.

It was suggested in this study that B1-SAPG-INS bound specifically to SGLT-1 and decreased BGL both in normal and diabetic rats. The mechanism of the hypoglycemic effect of B1-SAPG-INS, however, was not clear. The specific binding of B1-SAPG-INS to SGLT-1 increased the concentration of INS in close proximity to the intestinal wall which resulted in enhancement of its intestinal permeability. It has been reported that E-2078, a dynorphin-like analgesic peptide, was transported through the blood-brain barrier by an absorptive-mediated endocytosis mechanism [24]. In this case the positively-charged peptide interacted with the negatively-charged cell surface and was absorbed. The peptide was then transported into the cell by endocytosis. There is another endocytosis mechanism which is known as receptor-mediated endocytosis (RME). In RME the receptor is generally taken up into the cell together with the ligand binding to it and the later is recycled on the cell surface. It has been reported that the Na^+-dependent D-glucose transporter (SGLT-1) is located on the cell surface and is not recycled between the cell membrane and intracellular compartment [25]. It is, therefore, conceivable that B1-SAPG-INS may interact with the SGLT-1 and bind to BBMVs which may enhance the internalization of B1-SAPG-INS by endocytosis.

In conclusion, it is suggested that B1-SAPG-INS is adsorbed specifically onto the glucose transporter of intestinal BBM. This specific adsorption may be involved in the mechanism of the enhanced hypoglycemic effect of B1-SAPG-INS in both normal and diabetic rats.

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