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Erythritol-based dry powder of glucagon for pulmonary administration

Kosuke Endo[a], Satoko Amikawa[a], Asami Matsumoto[a], Norio Sahashi[b], Satomi Onoue[a,c,*]

[a] *Pharmaceutical Division, Ito Life Sciences Inc., 1-2-1 Kubogaoka, Moriya, Ibaraki 302-0104, Japan*
[b] *Department of Biology, Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan*
[c] *Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan*

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Abstract

Glucagon, a key regulatory element of glycogen metabolism, is known to be effective in the clinical treatment of hypoglycemia and the maintenance of normal circulating glucose levels in patients with total pancreatectomy, however the clinical use of this gut hormone has been restricted to parenteral administration. In this investigation, we prepared dry powder dosage forms of glucagon, which were formulated by mixing micronized glucagon particles and excipients with larger carrier particles. To achieve alveolar deposition for subsequent systemic absorption, a dry powder inhalant (DPI) of glucagon was size-reduced to a mass median diameter between 1 and 6 μm, as measured by laser diffraction analysis. The use of erythritol as both excipient and carrier in DPI of glucagon resulted in high and reproducible flowability and dispersibility of the powder mixtures, and therefore it provided a low dosing of the active substances. Distinct transpulmonary absorption of glucagon was confirmed after intratracheal administration of the glucagon dry powder to anesthetized rats, as evidenced by the increase in the blood glucagon and blood sugar levels. These results suggested the usefulness of an erythritol-based powder form of glucagon for systemic administration.

Keywords: Dry powder inhalant; Erythritol; Glucagon; Carrier; Excipient; Pulmonary absorption

1. Introduction

Glucagon is a 29-amino acid peptide and a member of a highly homologous family of biologically active peptides including secretin, vasoactive intestinal peptide, and pituitary adenylate cyclase-activating polypeptide (Miyata et al., 1989; Vaudry et al., 2000).

* Corresponding author. Present address: Analytical Research and Development, Pfizer Global Research and Development, Nagoya Laboratories, Pfizer Japan Inc., 5-2 Taketoyo, Aichi 470-2393, Japan. Tel.: +81 569 74 4855; fax: +81 569 74 4748.
E-mail address: satomi.onoue@pfizer.com (S. Onoue).

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The primary physiological role of glucagon, together with insulin, is the maintenance of normal glycemia (Gelling et al., 2003). The liver has a central role in handling absorbed nutrients and in the regulation of hepatic glycogen disposability, and this requires a high density of glucagon and its specific receptors in the liver (Hansen et al., 1995). In addition, glucagon has a relaxation effect on the smooth muscle of the stomach, duodenum and colon, therefore this gut hormone is clinically used for upper gastrointestinal radiologic examinations and barium enemas (Mochiki et al., 1998). Additional actions of glucagon may include the promotion of insulin secretion (Pipeleers et al., 1985), the regulation of lipolysis (Hagen, 1961), the secretion of catecholamines (Onoue et al., 2001), and the suppression of feeding (Geary, 1999). Although it is difficult for totally pancreatectomized patients to maintain live function due to the lack of pancreatic hormones including glucagon, the administration of exogenous glucagon has been expected to be effective in promoting the metabolism of lipids and amino acids (Hirota et al., 1989).

Although glucagon exhibited these interesting biological activities that may be effective in the clinical treatment of diabetes, there is at least one serious problem in its clinical application. Generally, peptide/protein is highly sensitive to digestive biogenic enzymes (Hupe-Sodmann et al., 1997), hence its administration is usually restricted to injection, nasal administration, or other mucosal adsorption systems (Teshima et al., 2002). To improve the ease and safety of administration, a large number of drug delivery systems have been investigated which facilitate clinical treatment, especially for some chronic diseases. Among them, drug delivery with a dry powder inhalant (DPI) is known as the method for topical administration to the lung, thus avoiding the first pass effect and gastric digestion. This DPI system may be applicable to the pulmonary administration of glucagon in the clinical treatment.

The aim of the present study was to develop an effective DPI system for glucagon. We investigated a dry powder formulation for inhalation in order to clarify its optimal conditions for glucagon, focusing on the type of carrier and excipient. Micronized lactose has been used as a carrier molecule for DPI, and previous studies showed that other sugars, including mannitol and sorbitol, also be applicable to DPI (Tee et al., 2000). Although the attention has been drawn to erythritol, a novel excipient, due to its pharmacological characteristics such as low calories and low hygroscopicity (Noda et al., 1994), its application to DPI has never been demonstrated. In this context, we also prepared and evaluated the peptide-containing DPIs with the use of erythritol as carrier and/or excipient. These examinations led us to the successful development of an efficient DPI with high emission and an increased respirable fraction (RF) value. In addition, we also confirmed the bioavailability of DPI for the pulmonary administration of glucagon, glucagon-DPI, as evidenced by the fact that the glucagon-DPI caused an increase in the plasma glucagon and glucose concentrations in rats.

2. Materials and methods

2.1. Chemicals

Glucagon was constructed on MBHA-resin with the solid-phase *t*-Boc strategy employing optimum side-chain protection as reported previously (Onoue et al., 2002). The peptide was removed from the resin by HF treatment, and the product was purified by column chromatography on a C-18 column. The homogeneity was assessed by RP-HPLC on the TSK-gel ODS-120T (TOSOH, Tokyo, Japan) and by amino acid analyses on the amino acid analyzer L-8500 (HITACHI, Tokyo, Japan). Their molecular weights were confirmed on a matrix-assisted laser desorption ionization-time of flight mass spectrometer (Kratos, Manchester, UK). Insulin and salmon calcitonin were purchased from American Peptide Company (Sunnyvale, CA). Pharmatose® 325 M and erythritol were supplied by DMV (Veghel, The Netherlands) and Nikken Chemicals (Tokyo, Japan), respectively. Erythritol-carrier, with the median volume diameter of 36 μm, was also kindly supplied from Nikken Chemicals. Lactose for excipient was purchased from Yoshida Pharmaceutical Co. Ltd. (Tokyo, Japan).

2.2. DPI preparation

Peptides and excipients were first ground to fine powders with a pestle and mortar and then milled with an A-O JET MILL (Seishin Enterprise Co. Ltd., Tokyo, Japan) at a pusher nozzle pressure and grinding nozzle

pressure of 0.65 and 0.60 MPa, respectively. The ratio of glucagon to excipient was 1:100, and that of insulin to excipient was 1:20. The micronized materials were decompounded with carrier particles in a plastic bag for 3 min, and the obtained dry powders of peptide/protein were stored in a vacuum desiccator until tested.

2.3. Measurement of particle size by laser diffraction

The particle size distribution of the micronized dry powder and/or carrier particle were determined by a laser diffraction particle size analyzer (LMS-30, Seishin Enterprise Co. Ltd., Tokyo, Japan) with dispersion by dry spraying at a pressure of 0.20 MPa.

2.4. Scanning electron microscope (SEM)

The blends composed of fine particles and carrier were coated with gold (thickness ca. 0.23 nm) on Ionsputter E-1020 (HITACHI, Tokyo, Japan). The samples were examined by SEM (mini SEM MSM-2, HITACHI, Tokyo, Japan) operating on 15 kV and equipped with a secondary electron detector.

2.5. Powder dispersion by cascade impaction

The cascade impactor study (Andersen sampler, AS200, Sibata, Tokyo, Japan) was carried out according to the *Aerosols/Physical Test and Determinations* in USP23. Briefly, the blends were filled into a No. 2 hard capsule of hydroxypropyl-methylcellulose (Shionogi Qualicaps Co. Ltd., Nara, Japan), and the capsule was installed in a Jethaler® (Hitachi Unisia, Kanagawa, Japan) powder inhaler. The dry powder formulations (40 mg) were dispersed via the device with an inspiration rate of 28.3 l/min for an inhalation time of 10 s 10 times, and the collection stages of the impactor (stages 0–7) were washed with 0.1% trifluoroacetic acid solution. The peptide concentrations were measured by RP-HPLC equipped with a fluorescence detector RF-535 (Shimadzu, Tokyo, Japan). The mobile phase of water/acetonitrile (65:35, v/v) containing 0.1% TFA was degassed and pumped through a TSK gel ODS-120T column (4.6 mm i.d. × 250 mm, TOSOH, Tokyo, Japan) at a flow rate of 1.0 ml/min, and the column temperature was maintained at 40 °C.

2.6. Animals and test procedure

Male Sprague-Dawley rats (ca. 300 g), starving for 16–24 h, were anesthetized with pentobarbital (40 mg/kg, i.p.) during the experiments. Glucagon-DPI that was composed of lactose-excipient and lactose-carrier, 4.1 mg/body (11.6 μg-glucagon/body), was administered with an apparatus for intratracheal administration (PD-3, INA Research Inc., Nagano, Japan). The amount of dry powder remaining in the apparatus after inhalation was determined by RP-HPLC equipped with a fluorescent detector RF-535 (Shimadzu, Tokyo, Japan), and then the dose of inhaled glucagon was estimated by deducting the remaining glucagon from its total amount in the dry powder of glucagon. Samples of blood (500 μl) were collected from the tail vein at various times during the first 1.5 h after the dry powder of glucagon was administered, and then centrifuged at 4 °C to separate the plasma.

2.7. Determination of glucagon and blood sugar

The determination of the glucagon level was performed by the ELISA method using human anti-glucagon monoclonal antibody (Wako, Osaka, Japan) and purified peroxidase-labeled goat anti-rabbit IgG (Wako). Plasma glucose was determined by the glucose oxidase method using Express Plus (Ciba-Corning, Tokyo, Japan).

2.8. Statistical analysis

For statistical comparisons, one-way analysis of variance (ANOVA) with pairwise comparison by the Fisher's least significant difference procedure was used. A *P*-value of less than 0.05 was considered significant for all analyses.

3. Results and discussion

3.1. Stably dispersed particles of glucagon

Peptides (pharmaceutical products) and lactose-excipients were initially mixed well, and micronized in a jet mill. After preparation of the micronized formulation, these fine particles were mixed with a carrier molecule (Pharmatose®), consisting of lactose, which

resulted in a dry powder formulation of glucagon for inhalation. HPLC analysis of glucagon before and after miconization displayed the almost similar purity of glucagon (data not shown), suggesting that the jet milling have no effect on the stability of glucagon. The carrier molecule could inhibit the self-aggregation among the fine particles, and it maintained a certain degree of flowability and uniformity (Prime et al., 1997). As shown in Fig. 1, laser diffraction analysis indicated that the carrier had a volume median diameter of 60 μm (Fig. 1B), and that of the micronized glucagon powder and excipient was estimated to be 2.5 μm (Fig. 1A). With respect to the dry powder formulation, it showed a wide ranging particle size distribution with two obvious peaks at approximately 2.6 and 64 μm that corresponded to the particle size distribution of the micronized materials and carrier molecules, respectively (Fig. 1C). These data suggested that the dry powder formulation of glucagon could be dispersed into a cloud of fine particles and carrier at a pressure of 0.15 MPa or higher. In addition to glucagon, insulin could also be used as a pharmaceutical agent for this dry powder formulation, and their DPI showed almost the same dispersion and size distribution as those of glucagon in laser diffraction analyses (data not shown). Exchange of lactose-excipient/carrier to those of erythritol in the DPI had no significant effect on their flowability and dispersibility, and laser diffraction analysis of erythritol-based DPI show the similar size-distribution as compared with lactose-based DPI (data not shown). SEM photographs of the carrier (Fig. 2A) and dry powder formulation of glucagon (Fig. 2B) showed the localization of micronized particles of the glucagon on the surface of the carrier particles. It can be seen that the attachment of glucagon and excipient on the lactose-carrier was homogeneous, and Fig. 2C and D indicate that erythritol would also be applicable as the carrier molecule in the preparation of DPI.

Peptides/proteins were well-known to be susceptible to the proteolytic degradation in the stomach or other digestive systems (Wallace and Lasker, 1993), and their dosage forms were often restricted to injection because of their low bioavailability by oral administration. Furthermore, therapeutic peptides, including insulin, glucagon, growth hormone, and glucagon-like peptide-1, at high concentrations led to the pharmacological consequences of non-covalent solution structural phenomena such as alternative molecular conformations and self-association, causing cytotoxicity in some neurons (Liu and Schubert, 1998). These insoluble products have been attributed to the formation of partially unfolded intermediates with an exposed hydrophobic region that drives the aggregation towards a pharmaceutically undesired form. Glucagon displayed an unfolded random structure in high polar and/or acidic conditions, and then hydrophobic side groups partially tended to huddle together to avoid the aqueous environment (Beaven et al., 1969). We believe that these serious problems are easily avoidable when therapeutic peptides are subjected to a dry powder formulation for inhalation.

3.2. Cascade impactor study of several dry powder formulations

Cascade impactor analyses of DPIs showed the in vitro deposition of the pharmaceutical peptide/protein, and the percentage of fine particles in stages 2–7 of

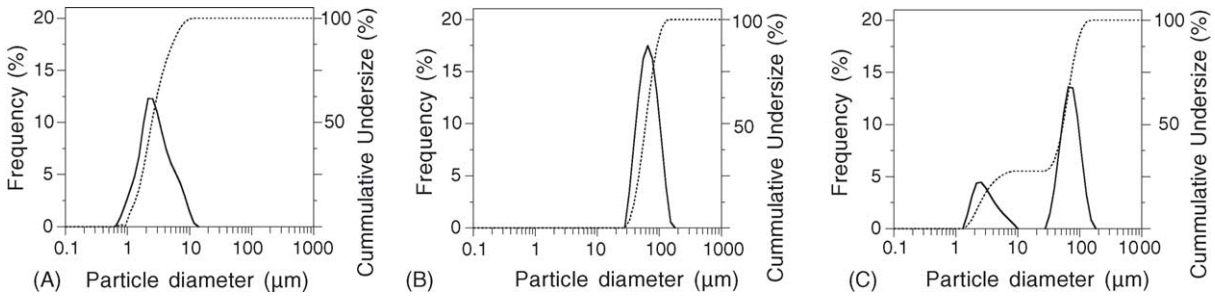

Fig. 1. Laser diffraction analysis of glucagon-DPI and its components. DPI and its components were dispersed by dry air at a pressure of 0.15 MPa, and their size-distribution and mean particle sizes were estimated. (A) Micronized powders of glucagons-lactose mixture; (B) lactose-carrier (Pharmatose®); (C) glucagon-DPI.

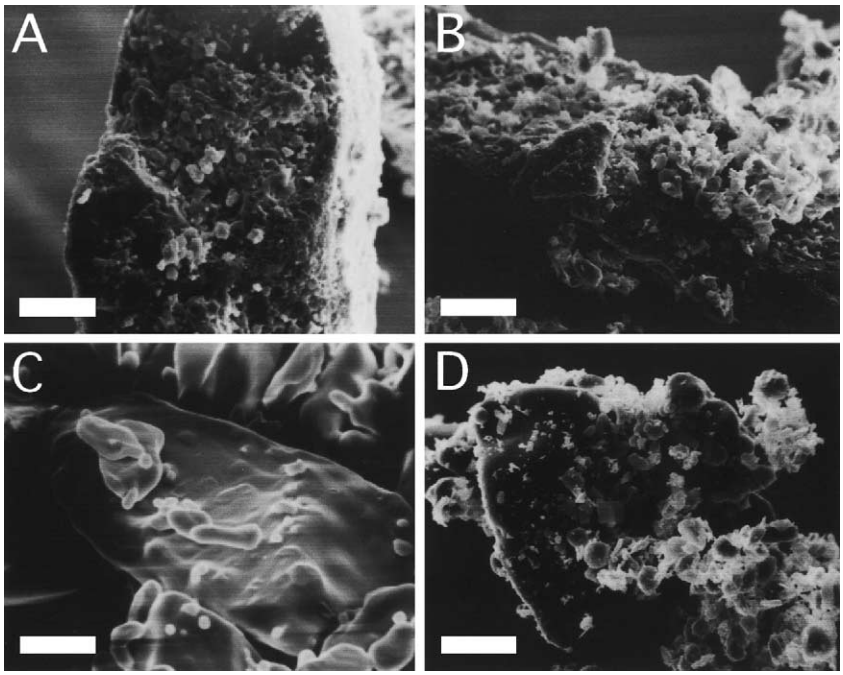

Fig. 2. Scanning electron micrograph images of glucagon-DPIs and carriers. Fine particles were composed of a jet-milled glucagon-erythritol mixture. (A) Lactose-carrier; (B) glucagon-DPI composed of lactose-carrier; (C) erythritol-carrier; (D) glucagon-DPI composed of erythritol-carrier. Scale bars indicate 10 μm.

the delivered dose was considered to be the respirable fraction (RF) value. In the present study, we selected Jethaler® as the inhalation device, and it was directly connected to the cascade impactor for the analyses of deposition of dispersed pharmaceutical agents according to the protocol in USP23. By dispersing via a Jethaler®, fine particles and carriers, the components of DPIs, were separated immediately, producing an aerosol of peptide particles. The device-aided dispersion of the dry powder was significantly affected by its encapsulated volume, and the RF value of DPI without pharmaceutical agents became lower as the encapsu-

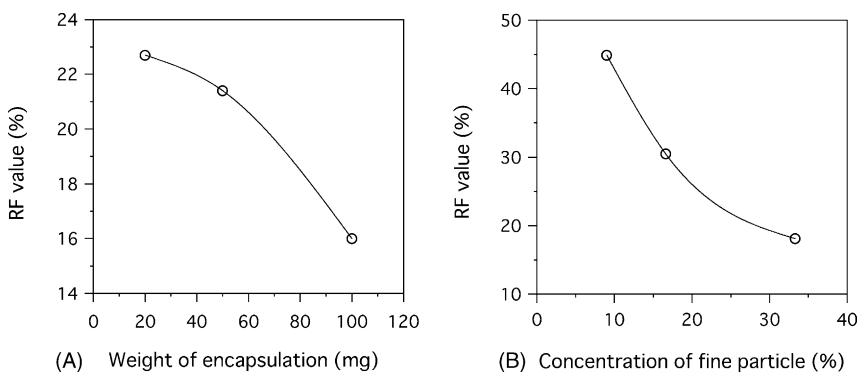

Fig. 3. Dispersibility of DPIs with various formulations. The tested DPIs were formulated with lactose-excipient and lactose-carrier, and each encapsulated DPI was inhaled via Jethaler® 10 times. RF values were measured by cascade impactor analysis at various encapsulated volumes (A), or at various concentrations of adhesive fine particles in the same emcapsulated volume (40 mg) (B).

lated volume increased (Fig. 3A). The RF value of DPI at the encapsulated weight of 100 mg was 1.4-fold less than the value at 20 mg, possibly due to the lack of a sufficient air stream for dispersion in the capsule.

In addition to the encapsulation, we investigated the influence of the concentration of fine particles in the blend (w/w) on the dispersion of dry powder from the inhalation device. As shown in Fig. 3B, a reduction of the concentration of fine particles in the blend resulted in a significant increase in its RF value, and the RF value at the concentration of 9% was 2.5-fold higher compared to the dry powder at the fine particle-concentration of 33%. This may reflect the self-association of fine particles on the surface of the carrier molecules in the presence of an excess of fine particles. Based on these results, a formulation of dry powder for high dispersion may be optimized when both the encapsulation and concentration of fine particles in the blend were reduced. On the contrary, an over-reduction of these parameters may lead to several pulmonary administrations being required due to its low dosage per capsule. Therefore, we decided on an encapsulated weight of 40 mg in the capsule, and a concentration of fine particles in the blend of 28.5%, and this formulation system was applied to the dry powders of therapeutic peptides used in the followed investigation.

3.3. Usage of erythritol as an excipient and carrier to disperse the dry powder

In addition to glucagon, we tried to prepare dry powder formulations of other therapeutic peptides including insulin, salmon calcitonin, and all of them were applicable to the dry powder inhalation since their dry powder formulations had high dispersion in the cascade impactor analyses. The RF values of the dry powders consisting of these other therapeutic peptides are shown in Table 1, and those of glucagon, insulin, and salmon calcitonin with lactose-carrier were estimated to 6.4%, 14.6%, and 29.9%, respectively. Interestingly, the conversion of carrier from lactose to erythritol resulted in a significant increase in RF values as follows: glucagon, 33.9%; insulin, 22.2%; salmon calcitonin, 61.8%.

As well as the application of erythritol as a carrier molecule in DPI, its use as an excipient also led to a significant increase in the RF value for the dry powder formulation of glucagon. The weight distribution of the powder deposited in each stage of the cascade impactor is shown Fig. 4A and B. With respect to the deposition of the glucagon powders in each stage of the cascade impactor, the lactose-excipient/lactose-carrier blend was mainly deposited in stage 0 (Fig. 4A), whereas the blends containing erythritol showed a decreased powder deposition in stage 0 (Fig. 4B), result-

Table 1
RF values from cascade impactor analysis using lactose-excipient particles and lactose/erythritol-carrier particles

Pharmaceutical agents	RF value (% of total)	
	Lactose	Erythritol
Glucagon	6.4	33.9
Insulin	14.6	22.2
Salmon calcitonin	29.9	61.8

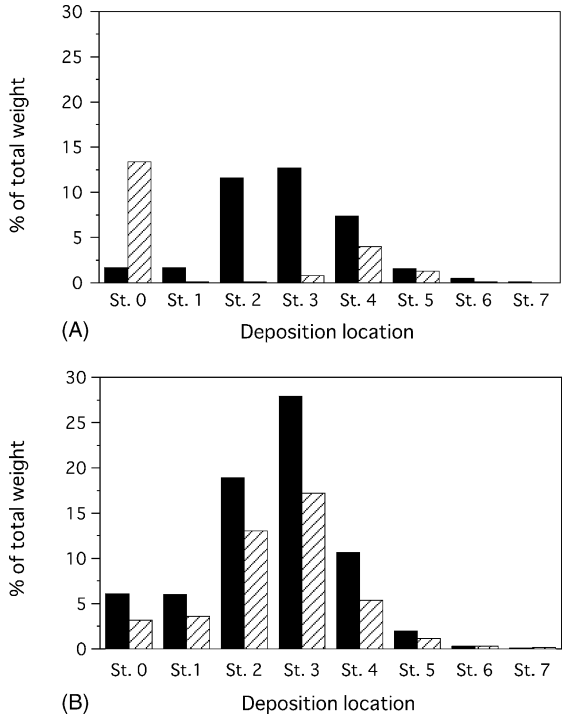

Fig. 4. Cascade impactor analysis of glucagon-DPIs. The tested blends are composed of micronized glucagon-erythritol mixtures and carrier. The tested DPI (40 mg) was dispersed via Jethaler® 10 times. (A) The powders were made with lactose-excipient and lactose-carrier (hatched bar); and with lactose-excipient and erythritol-carrier (filled bar). (B) The powders were made with erythritol-excipient and lactose-carrier (hatched bar); or with erythritol-excipient and erythritol-carrier (filled bar).

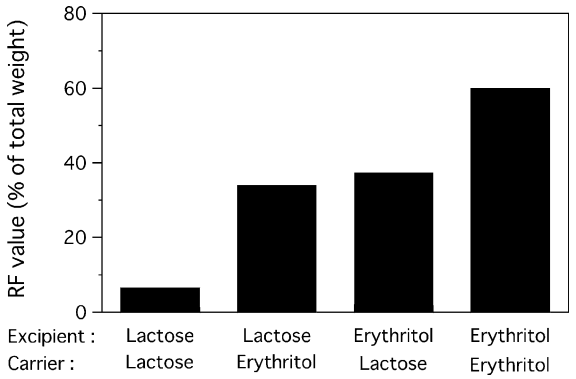

Fig. 5. In vitro deposition of glucagon-DPI on different types of carrier and excipient. Each excipient and carrier used in glucagon-DPI was lactose or erythritol. In cascade impactor analysis, each encapsulated DPI (40 mg) was dispersed via Jethaler® 10 times, then RF values for glucagon-DPI were evaluated by determining trapped glucagon powders in stages 2–7.

ing in a high RF value (Fig. 5). Thus, the dispersibility of dry powders seemed to be dependent on the excipient and carrier used in the formulation of the dry powder, and we confirmed the best performance with the glucagon formulation using erythritol as both the carrier and excipient (Figs. 4B and 5).

It is well-known that the physical properties of carrier particles strongly affect the RF value, in particular, a reduction of its roughness on the surface of the carrier substrate facilitates the separation of the drug particles from the carrier (Zeng et al., 2000). It has also been reported that an adhesion and interactive force of lactose with a drug depends on its surface morphology (Kawashima et al., 1998). The SEM images from the carriers composed of erythritol and lactose suggested that the erythritol-carrier has a smoother surface compared to the lactose-carrier, and this observation was consistent with our result showing that high dispersibility was confirmed in the erythritol-based DPIs. On the basis of these observations, we believe that this physicochemical property of erythritol may contribute partly to the fine dispersion of erythritol-based DPI. In addition to the application of erythritol as a carrier molecule, it is considered that erythritol is suitable as an excipient from the result showing that the erythritol-excipient blend increased the RF value (Figs. 4B and 5).

Furthermore, there are some beneficial characteristics of erythritol when used for excipients and carriers in DPIs as follows: (1) sweeter without calories; (2) high stablility; (3) low-hygroscopicity (Noda et al., 1994). Erythritol has no effect on the blood sugar level and insulin secretion, so it may be well suited as a therapeutic agent especially for treating diabetes (Ishikawa et al., 1996; Noda et al., 1994). An additional advantage that may be gained from its use as a carrier and excipient is an improvement in taste (Noda et al., 1994). In this context, these characteristics of erythritol supported its efficacy as the carrier and excipient in DPI systems.

3.4. Pulmonary administration of glucagon-DPI

After intratracheal administration of glucagon-DPI, the concentration of plasma glucagon was measured by enzyme-immuno assay, and plasma glucagon concentration versus time profiles are shown in Fig. 6. Inhaled glucagon produced readily detectable plasma glucagon concentrations (Fig. 6A), as did the injected glucagon (data not shown). The concentration of glucagon in the plasma reached the maximum level after 15 min, and decreased gradually to the same level as starting point after about 1 h. The bioavailability of glucagon (11.6 μg/body) after intratracheal administration was determined to 2.5% as compared with that of glucagon after injection. A significant increase in blood glucose level was confirmed in all subjects tested in 15 min (Fig. 6B). This is consistent with previous reports regarding the biological activity of glucagon (Teshima et al., 2002).

Transpulmonary administration of peptides and proteins can be expected to lead to higher rates of systemic absorption than other nonactive routes, because the alveolar epithelium where absorption takes place is thin and has a large surface area. Several hypotheses were proposed for the absorption mechanism (Gumbleton, 2001; Patton, 1996; Yu et al., 1996) and the hypothetical model for alveolar absorption of macromolecules varies depending on their molecular size. For small macromolecules (<40 kDa), paracellular and transcytotic mechanisms may both play roles in epithelial transport, whereas transcytosis may be the dominant transport mechanism across the epithelia for large macromolecules (>40 kDa). According to these hypotheses, the inhaled glucagon is absorbed in the alveoli mainly through paracellular and endocytotic pathways, and is then maybe transported into the

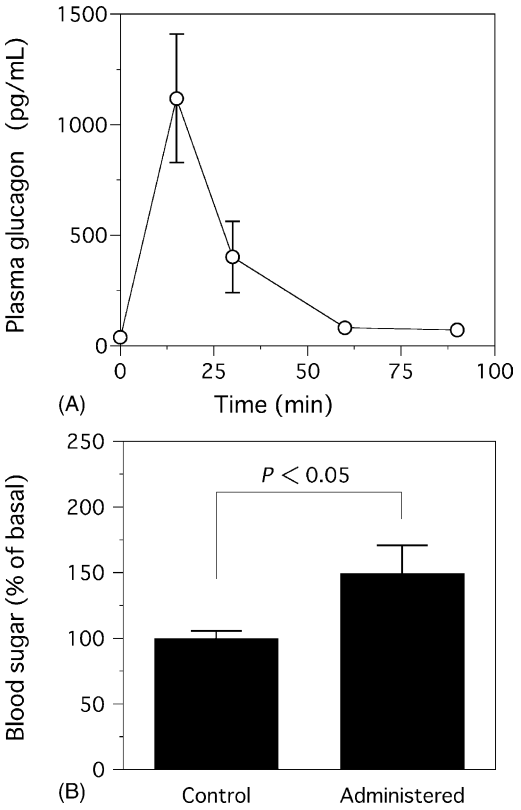

Fig. 6. The effect of the dry powder form of glucagon. Glucagon-DPI, 5 mg/body (11.6 μg-glucagon/body), was administered. (A) The concentrations of plasma glucagon in three normal rats following the administration of glucagon-DPI. Data represented are the mean ± S.D. of three measurements. (B) Blood sugar level 15 min after administration of glucagon-DPI. Mean blood sugar level in a non-treated subject was regarded as basal. Experiments were performed three times.

pulmonary veins, the termini of which are connected with arterial flow.

This study suggested that glucagon could be administered systemically using DPI system. We believe that dry powder administration is suitable for daily clinical treatment because of its easy handling, so the application of this system to glucagon may lead to novel dosage forms of glucagon for the maintenance of homeostasis, the treatment of hypoglycemia (Pontiroli et al., 1989; Rosenfalck et al., 1992), and the improvement of metabolic status and fatty liver in patients with pancreatectomy (Boden et al., 1980; Muller et al., 1979, 1983). In particular, the patients with pancreatectomy have to take glucagon frequently and, in this context, glucagon-DPI may be an effective formulation for the administration of glucagon. Moreover, the glucagon-DPI might replace injection on the direction of the current use of glucagon, i.e. diagnosis of glycogen storage disease and insulinoma, catecholamine release stimulation test (diagnosis of pheocromocytoma), cure of episodic severe hypoglycemia, and so forth.

4. Conclusion

This is, to the best of our knowledge, the first report demonstrating that pulmonary administration of glucagon in a dry powder formulation can lead to systemic absorption and the stimulation of glycogen metabolism. The present study indicated that the use of erythritol, as a carrier and/or excipient, enhanced the dispersion of micronized peptide/protein, possibly resulting in high emission to the lung, the targeted organs for inhaled dry powder. This dry powder inhalation of glucagon is applicable to the clinical treatment of hypoglycemia, and the maintenance of normal circulating glucose levels in patients with total pancreatectomy.

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