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Novel dry powder inhaler formulation of glucagon with addition of citric acid for enhanced pulmonary delivery

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ABSTRACT

Glucagon, a gut hormone, is one of the key regulatory elements in glucose homeostasis, and is clinically used for treatment of hypoglycemia and premedication in peroral endoscopy. Dry powder inhaler (DPI) form of glucagon is believed to be a promising new dosage form, and the present study aimed to develop a novel glucagon-DPI using absorption enhancer for improved pharmacological effects. The cytotoxicity of citric and capric acids, the potential absorption enhancers, at 1 and 10 mM was assessed by monitoring extracellular LDH levels in rat alveolar L2 cells, and a concentration- and time-dependent release of LDH was observed in capric acid, but not in citric acid-treated cells. DPI form of glucagon containing citric acid was prepared with a jet mill, and laser diffraction and cascade impactor analyses of the newly developed glucagon-DPI suggested high dispersion and deposition in the respiratory organs with an emitted dose and fine particle fraction of 99.5 and 25%, respectively. Addition of citric acid in glucagon-DPI improved the dissolution behavior, and did not impair the solid-state stability of glucagon-DPI. Intratracheal administration of glucagon-DPI (50 μg-glucagon/kg body weight of rat) containing citric acid led to 2.9-fold more potent hyperglycemic effect in rats, as compared to inhaled glucagon-DPI without citric acid. Based on these physicochemical and pharmacological characterization, the dry powder inhaler of glucagon with addition of citric acid would be of use as an alternative to injection form.

1. Introduction

Glucagon is a peptide hormone of 29 amino acids and a member of the highly homologous family of biologically active peptides including secretin, vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating polypeptide (Unger et al., 1978). This gut hormone is secreted from the α-cells of the pancreatic islets in response to hypoglycemia, and the primary physiological role of glucagon, together with insulin, is the maintenance of normal glycemia (Gelling et al., 2003; Rodbell et al., 1971; Unger et al., 1978). Glucagon also exhibits 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). Clinically, glucagon is used for premedication in upper gastrointestinal radiologic examinations and barium enemas (Mochiki et al., 1998), clinical treatment of hypoglycemia, and the clinical diacrisis for insulinoma, growth hormone deficiency and hepatic glycogenosis. Recently, impairment of hepatic functions and metabolic disorders of lipids and amino acids are serious problems in patients with pancreatectomy, due to the lack of pancreatic hormones including glucagon (Hirota et al., 1989; Teshima et al., 2002). Administration of exogenous glucagon is necessary for these patients to improve metabolic status. In spite of numerous pharmacological effects of glucagon, its clinical dosage form is currently limited to only injection, due to poor oral bioavailability.

Recently, the systemic delivery of therapeutic peptides/proteins by inhalation has attracted considerable attention because it avoids the first pass hepatic metabolism and gastric digestion and because of the high absorption available from the lungs as compared to oral, nasal and transdermal administration (Codrons et al., 2003; Onoue et al., 2008). In inhalation therapy with the use of nebulizer, drugs should be first solubilized/suspended in an aqueous medium and subsequently aerosolized through a nebulizer. However, in the solution state, significant hydrolysis, aggregation and/or structural conversion were often observed in some therapeutic peptides/proteins, including glucagon, salmon calcitonin and

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insulin, which resulted in the production of several degradants and a decrease of pharmacological effects (Onoue et al., 2006, 2004b, 2007). Thus, some peptides/proteins are unstable in the solution state, and consequently the use of a nebulizer would be unsuitable for peptides/proteins-based inhalation therapy. To overcome these drawbacks, our group previously developed a novel dry powder inhaler (DPI) system of glucagon and VIP derivatives for topical administration to the lung (Endo et al., 2005; Onoue et al., 2007), showing high emission from a capsule and deposition in the airway systems. However, the intratracheal bioavailability of glucagon was reported to be less than 1% (Patton et al., 2004), so further improvement on the formulation of glucagon-DPI would be necessary before clinical application. Use of an absorption enhancer was proposed to facilitate the absorption of glucagon via the mucous membrane (Pontiroli et al., 1989), although the practical application of an absorption enhancer to the glucagon-DPI system has never been demonstrated.

The present study aimed to develop an effective and safe DPI system for glucagon with use of an absorption enhancer for improved biological activities. Capric and citric acids were chosen as absorption enhancers, since they have been applied to DPI formulations of therapeutic peptides/proteins with aim of improving absorption (Agu et al., 2001; Todo et al., 2001). In particular, citric acid was included in Exubera®, an inhalable insulin formulation (Edgerton et al., 2005), suggesting safety and feasibility of using citric acid for DPI formulation of therapeutic peptides/proteins. The cytotoxic effects of these additives were assessed on rat alveolar L2 cells. Glucagon-DPI, composed of carrier particles and micronized glucagon with absorption enhancer, was prepared with a jet mill. The physicochemical properties of glucagon-DPI prepared were also characterized by electron microscopy for surface morphology, laser diffraction for particle size distribution, cascade impactor for predicting *in vitro* pulmonary deposition, and dissolution test. Stability studies were also carried out to clarify the influence of acidic additives on chemical degradation of glucagon during storage. In addition, the hyperglycemic activities of glucagon-DPIs with different contents of absorption enhancer were evaluated after the intratracheal administration in rats.

2. Materials and methods

2.1. Chemicals

Human glucagon was synthesized by a solid-phase strategy employing optimal side-chain protection as reported previously (Merrifield, 1969). The carrier particle of DPI, Respitose® SV-003, was supplied by DMV (Veghel, The Netherlands). Citric acid, trifluoroacetic acid (TFA) and Tween 20 were purchased from Wako pure chemical industries (Osaka, Japan). Capric acid, Dulbecco's modified Eagle's medium (DMEM) and pentobarbital were purchased from Sigma (St. Louise, MO). Newborn calf serum and kanamycin sulfate were obtained from Invitrogen Japan (Tokyo, Japan).

2.2. Cell culture

Rat alveolar L2 cells, originally derived from type II pneumocytes of adult rat lungs, were obtained from the American Type Culture Collection (Rockville, MD). L2 cells were cultured in DMEM supplemented with 10% (v/v) newborn calf serum containing 1% kanamycin sulfate. The cultures were maintained in 5% CO_2/95% humidified air at 37 °C.

2.3. LDH assay

The L2 cells were seeded at 5×10^3 cells/well in 96-well plates (AGC TECHNO GLASS, Chiba, Japan) at least 72 h before the experiment and cultured in serum-free DMEM. Absorption enhancers at various concentrations were added to the cultures, and the extent of cell death was assessed by measuring the activity of lactate dehydrogenase (LDH) released from the dead cells. The level of LDH activity in the culture medium was determined using a commercially available kit, Wako LDH-Cytotoxic test (Wako Pure Chemical Industries), according to the manufacturer's directions. The total LDH activity was measured under the same conditions but in the L2 cells treated with 2% Tween 20.

2.4. DPI formulations of glucagon

Dry powder inhaler of glucagon (GLG-DPI) and glucagon with citric acid (GLG/CA-DPI) was composed of carrier particles (Respitose® SV-003) and jet milled-glucagon with additives. Briefly, glucagon, lactose and absorption enhancer were firstly mixed with a pestle and mortar, and then milled with an A-O JET MILL (Seishin Enterprise Co. Ltd., Tokyo, Japan) at pusher nozzle pressure and grinding nozzle pressure of 0.60 and 0.55 MPa, respectively. The ratio (w/w) of glucagon to additives was 1:230. The micronized particles were mixed with a 10-fold amount of carrier particles, providing a DPI of glucagon with absorption enhancer. Glucagon-DPI without absorption enhancers was also prepared as control. DPIs were stored in vacuum desiccators until testing. The amount of glucagon in each DPI was determined using a Shimadzu class-VP HPLC system (Shimadzu, Kyoto, Japan) equipped with a Waters 2475 fluorescence detector (Waters, Milford, MA) that included a SCL-10Avp system controller, a SIL-10ADvp auto injector, a LC-10ADvp solvent delivery pump, a DGU-14A degasser, a CTO-10Avp column oven, and a SPD-10Avp UV-VIS detector. A CAPCELL PAK C18 MG II (particle size: 5.0 μm; column size: 3.0 mm × 150 mm; Shiseido, Tokyo, Japan) was used, and the column temperature was maintained at 40 °C. The fluorescence detector was adjusted for excitation wavelength at 290 nm and emission wavelength at 359 nm. The standard and samples were separated using a gradient mobile phase consisting of 0.1% TFA (A) and acetonitrile (B) with a flow rate of 0.5 mL/min. The gradient condition of mobile phase was 0–4 min, 30% B; 4–12 min, 30–100% B; 12–18 min, 100% B. Analytical method was validated partly according to ICH guideline Q2B "Validation of Analytical Procedures: Methodology". Linearity of the standard is good, with correlation coefficient 0.999 over a range of 8×10^{-9} to 4×10^{-8} M. The signal-to-noise ratio at the glucagon concentration of 8×10^{-9} M was found to be 13.9, and six injections at 4×10^{-8} M exhibited high reproducibility with the variation coefficient (%) of less than 1.

2.5. Scanning electron microscope (SEM)

Representative images of GLG/CA-DPI were taken using a scanning electron microscope, VE-7800 (KEYENCE, Osaka, Japan).

2.6. Particle size analysis by laser diffraction

Particle size of GLG/CA-DPI was measured by laser diffraction particle size analyzer (LMS-300, Seishin Enterprise Co. Ltd., Tokyo, Japan). GLG/CA-DPI was dispersed by dry air at a pressure of 0.2 MPa, and the particle size was determined. The particle size distribution was expressed as the volume median diameter and SPAN factor defined as SPAN = $(d_{90} - d_{10})/d_{50}$, where d_{10}, d_{50} and d_{90} are the particle diameters at 10, 50 and 90% of the cumulative volume, respectively. A high SPAN value indicates a wide size distribution.

2.7. Cascade impactor

The dispersibility of dry powder was assessed according to USP 29 ⟨601⟩ AEROSOLS with use of AN-200 system (Shibata Scientific Technology, Tokyo, Japan), consisting of vacuum pump, mass flow meter, and eight-stage Andersen cascade impactor. Briefly, dry powders were weighed into a JP No. 2 hard capsule of hydroxyproplyl methylcellulose (HPMC; Shionogi Qualicaps Co. Ltd., Nara, Japan), and the capsule was installed in a JetHaler® (Hitachi Unisia, Kanagawa, Japan) powder inhaler. The dry powder formulations (30 mg) were dispersed via the device with inspiration rate of 28.3 L/min for an inhalation time of 30 s × 5 times, and the collection stages of the impactor (stages 0–7) were washed with 0.1 M HCl. Glucagon in each solution was determined by HPLC as described in Section 2.4. Effective cutoff aerodynamic diameter for each stage was as follows: stage 0, 9 μm; stage 1, 5.8 μm; stage 2, 4.7 μm; stage 3, 3.3 μm; stage 4, 2.1 μm; stage 5, 1.2 μm; stage 6, 0.7 μm; stage 7, 0.4 μm. The fine particle dose (FPD) was defined as the mass of drug less than 5.8 μm (particles deposited at stage 2 and lower). The fine particle fraction (FPF) was calculated as the ratio of FPD to total loaded dose.

2.8. Dissolution test

Dissolution tests were carried out for 30 min in 10 mL distilled water with constant stirring at 50 rpm by magnetic stirrer. Fifty milligrams of glucagon-DPIs were weighed to the dissolution vessel. Samples were collected at the indicated times and filtered through the 0.22-μm filter. The filtrates were diluted with the same volume of 0.1 M HCl immediately after filtration. Experiments were run in triplicate. Glucagon in each solution was determined by HPLC as described in Section 2.4.

2.9. Stability study

The chemical stabilities were evaluated by storing ca 10 mg of GLG-DPI or GLG/CA-DPI at 25 and 40 °C for appropriate periods, up to 12 weeks in Platinous PR-2S thermo-hygrostat (Espec Corporation, Osaka, Japan). During storage, each vial was sealed with silicon rubber septum and aluminum crimp cap. Samples after storage were subjected to an HPLC analysis for determination of purity, as described in Section 2.4.

2.10. Drug inhalation and blood glucose measurement

Male Sprague–Dawley rats (6–8 weeks of age) (Japan SLC, Shizuoka, Japan) were housed two per cage in the laboratory with free access to food and water, and maintained on a 12-h dark/light cycle in a room with controlled temperature (24 ± 1 °C) and humidity (55 ± 5%). Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and glucagon-DPI (50 μg/kg glucagon) was intratracheally administered with an apparatus for intratracheal administration (DP-4, INA Research Inc., Nagano, Japan). At the appropriate time after dosing, the fasting blood glucose level was measured from tail-tip blood using glucose CII-test Wako (Wako Pure Chemical Industries). All procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethical Committee of University of Shizuoka.

2.11. Statistical analysis

For statistical comparisons, a one-way analysis of variance (ANOVA) with the pairwise comparison by 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. Cytotoxicity of absorption enhancers in rat alveolar cells

The pulmonary absorption of polar drugs, especially therapeutic peptides/proteins, is frequently limited due to their poor mucosal permeability. However, the use of absorption enhancers has been shown to improve the absorption characteristics of these chemicals (Onoue et al., 2008; Pontiroli et al., 1989). Previously, a number of absorption enhancers including glycyrrhizinic acid, surfactant, cyclodextrin, fatty acid, taurocholate, saturated polyglycolysed glyceride, trihydroxy bile salt and hyaluronic acid were proposed for the improvement of the bioavailabilities and pharmacodynamic responses of biotherapeutic agents, including insulin and calcitonin (Agu et al., 2001; Hooton et al., 2006; Imai et al., 1999; Onoue et al., 2008). In particular, the use of fatty acids, preferably capric acid, has been attempted in pharmaceutical industries for enhancing mucosal absorption of therapeutic peptides/proteins in small intestine, nasal and pulmonary tissues. In addition to fatty acids, citric acid appeared to be a potent absorption enhancer for insulin in dry powder inhalers (Todo et al., 2001), and it received regulatory approval for pulmonary administration (Maximal dose approved in Japan: 9.6 mg).

In the present investigation, citric and capric acids were chosen as absorption enhancers for the DPI form of glucagon, and the topical cytotoxic effects of citric and capric acids were initially assessed using rat alveolar L2 cells. The L2 cells, first isolated and cloned from adult rat alveolar epithelial cells, maintained the shape of type II alveolar pneumonocytes and retained the phenotype and functions of type II cells. Therefore, L2 cells have been utilized by several groups as a model for alveolar type II epithelial cells to study the responsiveness of lung type II cells to oxidative stress or toxic chemicals (Onoue et al., 2004a; Shi et al., 1994). The L2 cells were treated with citric or capric acid at the concentrations of 1 and 10 mM for 72 h, and the extent of cell death was assessed by the measurement of LDH released from dead cells, due to the loss of cell membrane integrity observed in both necrotic and apoptotic cells. The treatment of L2 cells with capric acid induced a concentration (1 and 10 mM)-dependent and exposure time (12–72 h)-dependent release of cellular LDH activity into the culture medium (Fig. 1). The extracellular LDH activity released by capric acid at a concentration of 10 mM for 48 h was equivalent to 29% of the total LDH activity in L2 cells. Citric acid was found to be less cytotoxic in L2

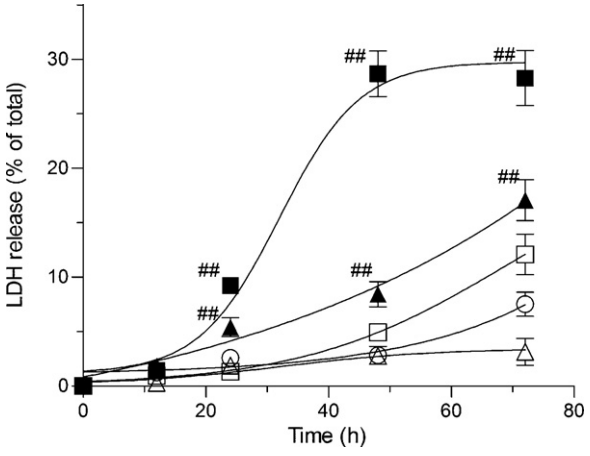

Fig. 1. Cytotoxicity of absorption enhancers in rat alveolar L2 cells. L2 cells were treated with 1 and 10 mM of capric and citric acids for the indicated periods, and extracellular LDH levels were measured. (○) Control (vehicle alone); (△) citric acid at 1 mM; (▲) citric acid at 10 mM; (□) capric acid at 1 mM; (■) capric acid at 10 mM. Each point represents the mean ± SE of four experiments. $^{\#\#}P < 0.01$ with respect to control.

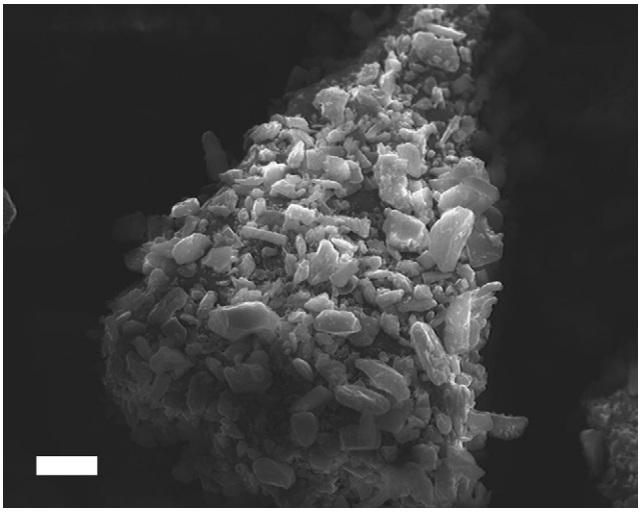

Fig. 2. Scanning electron microscopic images from GLG/CA-DPI. Scale represents 10 μm.

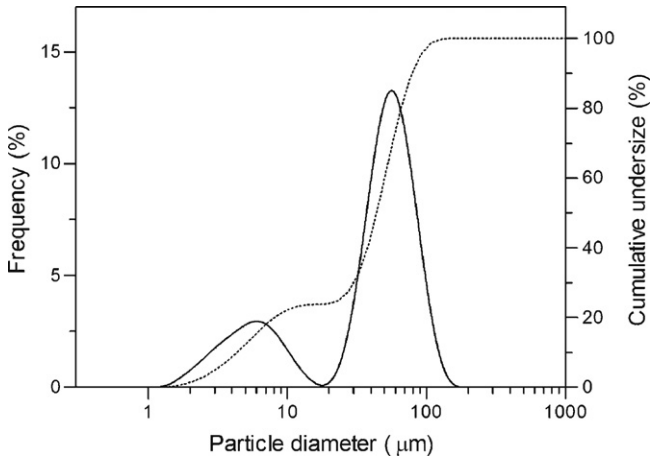

Fig. 3. Particle size distribution of aerosolized GLG/CA-DPI. Particle size and aerosolization efficiency of GLG/CA-DPI were evaluated by laser diffraction particle size analysis. GLG/CA-DPI was dispersed by dry air at a pressure of 0.2 MPa, and the size-distribution and mean particle size were estimated.

cells, as evidenced by 78% lower LDH release from L2 cells treated with citric acid at 10 mM for 48 h, as compared to capric acid at 10 mM. Although exposure of L2 cells to citric acid at 10 mM for 48 h resulted in slight LDH release (8.4% of the total LDH activity), no significant LDH leakage was observed in L2 cells treated with citric acid at 1 mM during the experimental period. Based on these data, taken together with previous reports, citric acid might have the ability to promote mucosal membrane absorption with minimal acute toxicity problems. However, the possible side effects following chronic use (10–20 years) are yet to be ascertained, and further studies on the safety of inhaled glucagon with citric acid are required in order to avoid serious undesired side effects.

3.2. Preparation and physicochemical properties of glucagon-DPIs

Because of their cohesive properties, micronized drugs typically have poor flow properties (Zheng et al., 2001) and tend to agglomerate. In most dry powder formulations, a carrier is used to improve the flowability and dispersibility of drugs, to facilitate the drug release from the inhaler and to enable deep lung deposition of the drug. The addition of a carrier also increases the volume of the inhalation powder, making the metering of the inhaled dose more accurate. In the present study, the sieved lactose carrier Respitose® was mixed with jet-milled particles of glucagon and citric acid, providing GLG/CA-DPI, a DPI form of glucagon. A SEM photograph of the GLG/CA-DPI indicated that the micronized glucagon particles adhered to the surface of the lactose carriers (Fig. 2). No significant aggregates of micronized particles were observed in the GLG/CA-DPI prepared, possibly due to addition of lactose carrier particles for stabilization. Without the lactose carrier, jet-milled glucagon-additive mixture seemed to be agglomerated, producing large particles (data not shown). These observations were consistent with our previous data on DPI forms of glucagon, insulin or salmon calcitonin without an absorption enhancer (Endo et al., 2005). Figure 3 shows a representative particle size distribution of aerosolized GLG/CA-DPI as measured by the laser diffraction method. According to the data obtained, GLG/CA-DPI could be dispersed into a cloud of fine particles and carrier at a pressure of 0.2 MPa or higher. Two peaks for the lactose–glucagon–citric acid blend and lactose carrier were observed, ranging from 1 to 11 and 11 to 110 μm, d_{50} of which were calculated to be 4.7 and 52.1 μm, respectively. The SPAN factor for the small particles was found to be 1.9, which could be indicative of the narrow size distribution of the micronized particles. These data indicated that the powders would be of a suitable size to avoid deposition by inertial impaction in the oropharyngeal cavity (Larhrib et al., 2003). Generally, glucagon is highly sensitive to oxidation and hydrolysis, however, after jet-milling process, degradants of glucagon were negligible in HPLC analysis of glucagon formulations (data not shown).

Currently, cascade impactor and twin impinger have been widely accepted as *in vitro* models of pulmonary deposition of particulate matter. In the present investigation, the inhalation properties of GLG/CA-DPI and its emission from the capsule were evaluated by cascade impactor connected with JetHaler®, an inhalation device (Table 1). The dose emitted from the capsule was found to be 99.5% of the nominal dose, and the emitted fine particles and carriers, the components of DPIs, could be separated immediately with the JetHaler®, producing an aerosol of fine glucagon particles. Aerosolized fine particles were trapped in each stage of the cascade impactor, and the FPF value of GLG/CA-DPI was calculated to be 25.0%. No significant differences in FPF values were observed between GLG-DPI and GLG/CA-DPI (data not shown), and the present results indicated that the use of citric acid in the DPI system of glucagon does not impair the inhalation properties of the glucagon inhalation powder.

Table 1
Distribution patterns of aerosolized GLG/CA-DPI in cascade impactor.

	Expected size of trapped particles (μm)	Glucagon (% of total)
Stage no.		
0	9.0–10.0	29.6
1	5.8–9.0	13.1
2	4.7–5.8	17.0
3	3.3–4.7	7.2
4	2.1–3.3	0.5
5	1.2–2.1	0.1
6	0.7–1.1	0.1
7	0.4–0.7	0.1
Fine particle fraction value (%)		25.0
Emitted dose (%)		99.5

Deposition pattern analysis of GLG/CA-DPI was conducted by cascade impactor connected to a JetHaler® with an airflow rate of 28.3 L/min. The fine particle fraction (FPF) was defined as the amount of powder with an aerodynamic size of less than 5.8 μm (particles deposited at stage 2 and lower) divided by the nominal dose and expressed as a percentage. The emitted dose means the percentage of drug emitted from the capsule.

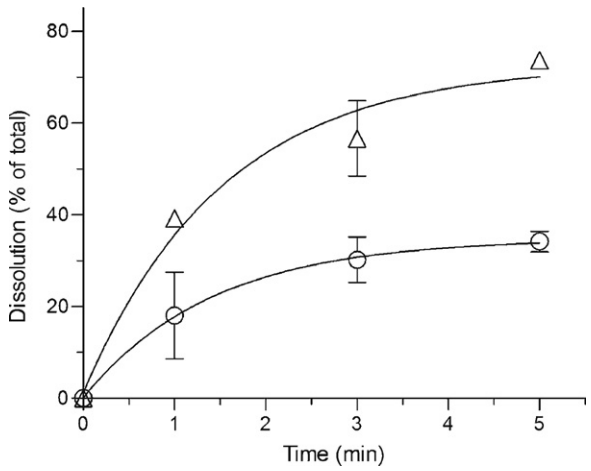

Fig. 4. Dissolution profiles of glucagon DPIs in deionized water. GLG/CA-DPI (△) and GLG-DPI (○). Each point represents the mean ± SD of four experiments.

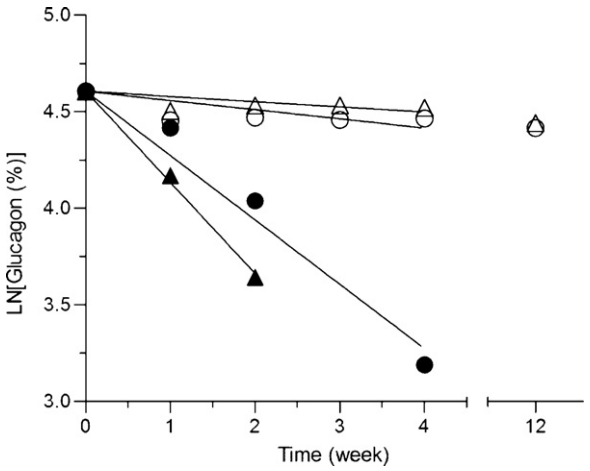

Fig. 5. Solid-state stability of glucagon-DPIs. Each DPI was stored at 25 and 40 °C for the indicated periods. (△) GLG/CA-DPI stored at 25 °C; (▲) at 40 °C; (○) GLG-DPI stored at 25 °C; (●) stored at 40 °C. Each point represents the mean ± SE.

3.3. Dissolution and stability studies on glucagon-DPIs

The isoelectric point of glucagon ranges from 7.5 to 8.5, so glucagon is poorly water-soluble in the neutral pH range but easily soluble in acidic condition. In this study, a dissolution test on glucagon-DPIs, including GLG- and GLG/CA-DPIs, was carried out to clarify the potential role of citric acid in the solubility of glucagon in water (Fig. 4). Although both DPIs immediately reached plateau in their dissolution profiles, GLG/CA-DPI showed an improved dissolution behavior in water, as compared to GLG-DPI. In contrast, neither DPI could reach the theoretical maximum level of dissolution. Generally, glucagon adheres to the surface of glass vessels, and this could be attributed to the loss of glucagon levels released. The results indicated that the addition of citric acid into DPI formulations might be effective for solubilization of glucagon in water. However, citric acid, contained in GLG/CA-DPI, might cause acid degradation of glucagon during long-term storage. In this context, solid-state stability studies on GLG/CA-DPI, as well as GLG-DPI, were conducted to assure sufficient chemical stability of glucagon in the DPI form even when citric acid is included as an absorption enhancer. Both DPIs were stored at 25 and 40 °C, resulting in time-dependent degradation of glucagon (Fig. 5). However, no significant degradation of glucagon was observed during storage at 4 °C (data not shown). A clear linear relationship was obtained according to the following equation: $\ln A = \ln A_0 - kt$, where A is the remaining peak area of glucagon, k is the slope and t is the time (h). Degradation was evaluated on the basis of the kinetic degradation constant k and the 10 and 50% life times ($t_{0.1}$ and $t_{0.5}$) with respect to initial drug concentration (Table 2). The degradation processes of glucagon in GLG- and GLG/CA-DPIs were highly accelerated with increasing temperature as evidenced by ca. 17- and 26-fold elevated degradation rates compared to DPIs stored at 25 °C. According to the stability data at 25 °C, there seemed to be no significant difference in stability between GLG- and GLG/CA-DPI. Based on these findings, citric acid might slightly enhance the dissolution of glucagon, possibly leading to improved pharmacological effects, and it would not affect the stability of glucagon during storage.

3.4. Pharmacological characterization of glucagon-DPIs

The primary physiological role of glucagon, together with insulin, is the maintenance of normal glycemia, and glucagon is responsible for stimulating hepatic glucose production, mediated by the cyclic AMP increase due to the activation of adenylate cyclase resulting from interaction of glucagon with a specific receptor expressed in the liver (Gelling et al., 2003; Rodbell et al., 1971; Unger et al., 1978). In this study, glucose levels in rats were monitored after inhalation of glucagon-DPIs, since the transition of pharmacological responses would reflect the effect of citric acid on the pulmonary absorption of inhaled glucagon. After intratracheal administration of GLG-DPI (50 μg-glucagon/kg body weight of rat as a single dose), a marked elevation of blood glucose was observed (Fig. 6A). The effect of GLG-DPI reached a maximum level of 165% of the basal level at 15 min, and disappeared at 90 min after inhalation. Inhaled GLG/CA-DPI, containing citric acid at doses of 50 μg and 1 mg/kg rat body weight, exhibited 42 and 104% enhancement of maximal blood glucose level compared to GLG-DPI, but the time to maximal glucose concentration seemed to be unchanged. In addition, prolonged duration of action was observed in rats treated with GLG/CA-DPI (1 mg-citric acid/kg body weight of rat). Values for area under the increased blood glucose-time curve of GLG-DPI, GLG/CA-DPI containing 50 μg- and 1 mg-citric acid/kg body were calculated to be 1630 ± 219, 2640 ± 325, and 4640 ± 381 mg·min/dL, respectively (Fig. 6B). According to the AUC values, addition of citric acid led to a significant improvement of pharmacological activities of glucagon in a concentration-dependent manner.

Table 2
Rate constants of thermal degradation for glucagon in DPI forms with or without citric acid.

	Temperature (°C)	Stability data		
		Degradation rate, k (week^{-1})	t_{10} (week)	t_{50} (week)
GLG-DPI	25	0.020	5.38	35.4
	40	0.333	0.32	2.08
GLG/CA-DPI	25	0.018	5.94	39.1
	40	0.472	0.22	1.47

Each parameter was calculated by computer-fitting.

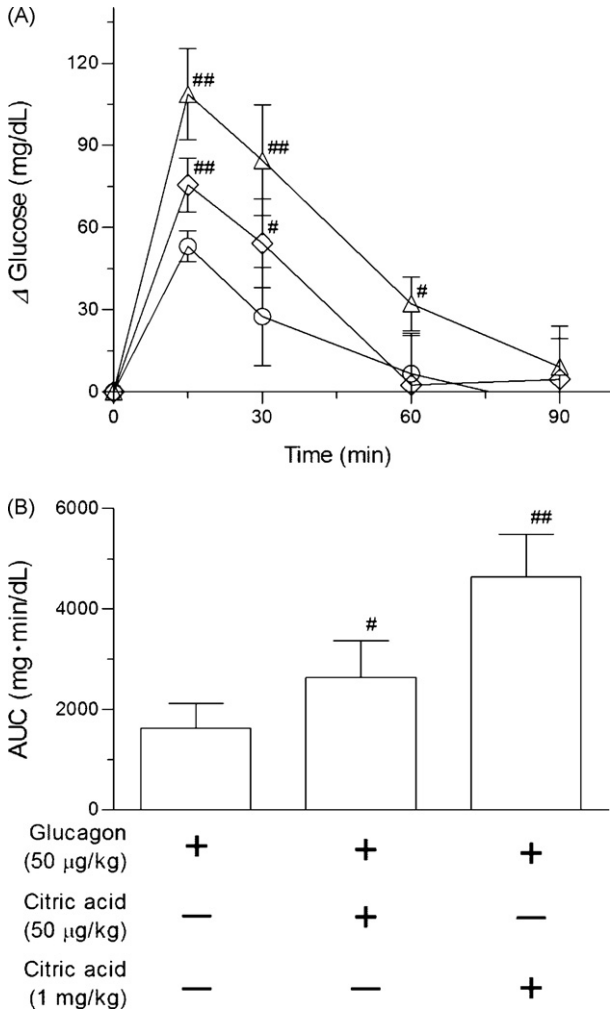

Fig. 6. Hyperglycemic activities of inhaled glucagon-DPIs with or without citric acid. (A) Increased level of blood glucose after intratracheal administration of glucagon-DPIs (50 μg-glucagon/kg body weight of rat). (○) GLG-DPI; (◊) with low amount of citric acid (50 μg-citric acid/kg body weight of rat); (△) with high amount of citric acid (1.0 mg-citric acid/kg body weight of rat, GLG/CA-DPI). (B) Area under the blood glucose-time curve from 0 to 90 min after intratracheal administration of glucagon-DPIs (50 μg-glucagon/kg body weight of rat). Data represents the mean ± SD of six experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ with respect to GLG-DPI without citric acid.

The marked enhancement of hyperglycemic activities would suggest the improved pulmonary absorption of glucagon after inhalation. These findings were consistent with previous reports, showing the accelerating function of citric acid on intestinal and nasal absorption of therapeutic peptides/proteins (Karasulu et al., 2008; Lee et al., 1999; Sinko et al., 1999). Citric acid appeared to be a potent absorption enhancer, possibly due to two main action mechanisms (Karasulu et al., 2008; Todo et al., 2001). First, citric acid could decrease the intracellular pH, with the intracellular acidosis tending to increase the calcium level through the decrease in ATP levels. These biochemical events could result in up-regulated permeability of the tight junction, followed by enhanced transport of therapeutic peptides/proteins through the paracellular pathway (Karasulu et al., 2008). In practice, stimulation of Caco-2 cells and jejunal preparations with citric acid resulted in marked reduction of transepithelial electrical resistance (Froment et al., 1989; Shah et al., 2004). Second, the mucosal pH decrease caused by citric acid might be critical for the inhibition of proteolytic degradation by enzymes. While the lungs are a far less hostile metabolic environment than the gastrointestinal tract, enzymes are still present in smaller amounts (Cryan, 2005). Therapeutic peptides/proteins may be enzymatically degraded intracellularly within macrophages and/or extracellularly by membrane-associated proteases and peptidases. The potential usefulness of enzyme inhibitors to improve absorption of biotherapeutic agents via the lung has been demonstrated (Agu et al., 2001). In addition to enzyme inhibitors (bacitracin, bestatin, soybean trypsin inhibitor, and chymostatin), some organic acids, including citric acid, exhibited potent inhibition of proteolytic activity against insulin, calcitonin and insulin-like growth factor-1 by pH-lowering mechanisms (Bai et al., 1995). Interestingly, citric acid acts as pH-modifier, as well as absorption enhancer, so that it could improve the dissolution property of glucagon, as observed in dissolution study (Fig. 4). The enhanced solubility of glucagon in water might lead to improved pharmacological effects. Based on these data, taken together with results from *in vitro* toxicity, physicochemical characterization and stability studies, the dry powder inhaler of glucagon with addition of citric acid would be effective for maintenance of normal circulating glucose levels in patients with total pancreatectomy.

4. Conclusion

In the present study, a novel DPI system of glucagon for inhalation therapy was developed with use of citric acid, an absorption enhancer. Micronized glucagon particles, prepared with a jet mill, showed suitable physicochemical properties for the inhalable dry powder, such as inhalation properties, dissolution and stability. As compared to glucagon-DPI without citric acid, significant improvement for the hyperglycemic effects was observed in glucagon-DPI containing citric acid, and this could be attributed to the enhanced absorption of glucagon by the addition of citric acid. These data would suggest a therapeutic benefit of the DPI form of glucagon with addition of citric acid as an alternative to the injection form of glucagon currently used in peroral endoscopy, clinical treatment of hypoglycemia, and clinical diacrisis for insulinoma, growth hormone deficiency and hepatic glycogenosis.

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