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RESEARCH PAPER

Inhalable Sustained-Release Formulation of Glucagon: *In Vitro* Amyloidogenic and Inhalation Properties, and *In Vivo* Absorption and Bioactivity

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ABSTRACT

Purpose The present study aimed to develop novel glucagon-loaded PLGA nanospheres without cytotoxic fibril formation for chronic glucagon replacement therapy.

Methods Glucagon-loaded nanospheres (GLG/NS) were prepared by an emulsion solvent diffusion method in oil, and a respirable powder formulation (GLG/NS-RP) was prepared with a jet mill. Physicochemical and inhalation properties of GLG/NS-RP were characterized, and pharmacokinetic behavior and hyperglycemic effect of intratracheally instilled GLG/NS-RP were evaluated in rats.

Results Although preparation of GLG/NS using glucagon solution at concentrations over 10 mg/mL led to significant formation of cytotoxic glucagon aggregates, glucagon solution at less than 5 mg/mL did not cause structural changes. Drug release behavior of GLG/NS showed a biphasic pattern with an initial burst and slow diffusion. Laser diffraction and cascade impactor analyses of GLG/NS-RP suggested high dispersion and deposition in the respiratory organs with a fine particle fraction of 20.5%. After the intratracheal administration of the GLG/NS-RP (200 μg glucagon/kg) in rats, glucagon was released in a sustained manner, leading to sustained hyperglycemic effects compared with those of normal glucagon powder.

Conclusion These data would suggest a therapeutic benefit of the newly developed GLG/NS-RP as an alternative to the injection form of glucagon currently used.

KEY WORDS cytotoxic fibril · dry powder inhaler · glucagon · PLGA · sustained release

INTRODUCTION

Glucagon, a member of the glucagon/secretin superfamily, is secreted from the α-cells of pancreatic islets in response to hypoglycemia, the primary physiological role of which is the maintenance of normal glycemia (1). Glucagon has been clinically used for premedication in barium enemas and clinical treatment of hypoglycemia (2). Recently, glucagon replacement therapy for patients with pancreatectomy has attracted attention since the lack of pancreatic hormones, including glucagon, sometimes causes hepatic dysfunctions and metabolic disorders of lipids and amino acids (3–5). In spite of the therapeutic potential, owing to poor oral bioavailability, the clinical dosage form of glucagon is currently limited to injection only, leading to limited clinical compliance. To overcome this drawback, our group previously

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developed a novel dry powder inhaler (DPI) system of glucagon for topical administration to the lung (6,7). In particular, addition of citric acid, an absorption enhancer, to DPI formulation of glucagon resulted in marked improvement of pharmacological effects, whereas no marked enhancements in duration of action were observed (7). For better clinical outcome from glucagon-based inhalation therapy for patients with total pancreatectomy, further improvement of the glucagon-based DPI system, focusing on prolonged duration of action, is necessary before clinical application.

Poly(lactide-co-glycolide) (PLGA) has been used for development of long-acting products of therapeutic peptides and proteins because PLGA is biodegradable and the rates of hydrolysis can be tailored to controlled drug release rates (8). Application of PLGA to the DPI system of glucagon might be a viable option for enhancing the duration of action; however, there appeared to be chemical degradation of the incorporated peptide/protein in PLGA devices. The susceptibility of PLGA ester bonds to nucleophilic attack and the accumulation of polymer hydrolysis products can lead to peptide and protein instability. PLGA hydrolysis, deamination at asparagine residues, peptide bond hydrolysis and acylation of protein primary amines occur for peptides and proteins in degrading PLGA systems (9). In addition, during preparation and/or storage of PLGA nanospheres, some amyloidogenic peptides/proteins might generate insoluble fibrils, owing to conformational transition from α-helix to β-sheet (10,11). These peptide/protein fibrils were found to be cytotoxic in several cells and tissues, followed by activation of apoptotic signaling pathways (10). Although *in vivo* toxicity of the peptide/protein fibrils in the pulmonary tissues has not been fully elucidated after chronic use, avoidance of degradation and fibril formation would be a key consideration for developing inhalable controlled-release formulation of glucagon with high safety.

The main purpose of the present study was to provide an efficacious and safe DPI system of glucagon using glucagon-loaded PLGA nanospheres (GLG/NS) for chronic glucagon-replacement therapy in patients with total pancreatectomy. Amyloidogenesis of glucagon in the GLG/NS was assessed by electron microscopy, circular dichroic (CD) spectral analysis and β-sheet specific dye, and the cytotoxicity of glucagon fibril was assessed in neuron-like PC12 cells and rat alveolar L2 cells. New respirable powder formulation of glucagon (GLG/NS-RP), composed of lactose carriers and micronized mixture of GLG/NS and erythritol, was prepared with a jet mill. The physicochemical properties of GLG/NS-RP were characterized by electron microscopy for surface morphology, laser diffraction for particle size distribution, cascade impactor for predicting *in vivo* pulmonary deposition and dissolution test. The hyperglycemic effect of GLG/NS-RP was evaluated after the intratracheal administration in rats, and the pharmacokinetic behavior of glucagon was also evaluated by EIA.

MATERIALS AND METHODS

Chemicals

Human glucagon was synthesized by a solid-phase strategy employing optimal side-chain protection as reported previously (12). The carrier particle of DPI, Respitose® SV-003, was supplied by DMV (Veghel, The Netherlands). Erythritol was supplied by Nikken Chemicals (Tokyo, Japan). Coconard MT was supplied by Kao (Tokyo, Japan). Hexaglycerin-condensed ricinolate (Hexaglyn PR-15) was supplied by Nikko Chemicals (Tokyo, Japan). Polyvinyl alcohol (PVA-403) was supplied by Kuraray (Tokyo, Japan). PLGA with an average molecular weight of 5,000 and copolymer ratio of $_{DL}$-lactide to glycolide of 50:50 as well as thioflavin T (ThT) were purchased from Wako (Osaka, Japan). Sorbitan monooleate (Span 80) was purchased from Tokyo Chemical Industry (Tokyo, Japan). WST-8 was purchased from Dojindo (Kumamoto, Japan). All other chemicals were purchased from commercial sources.

Glucagon-Loaded PLGA Nanospheres

The GLG/NS were prepared using an emulsion solvent diffusion method in oil (13). Briefly, glucagon, dissolved in 0.01 M NaOH, was added to 2 mL of acetonic solution, containing PLGA (40 mg) and Span 80 (40 mg), at a final glucagon concentration of 1, 5 or 10 mg/mL. The acetonic polymer solution was emulsified into a mixture of hexane (8 mL) and Coconard containing 2% (w/w) Hexaglyn (12 mL) using a high shearing homogenizer (Physcotron, Nition, Japan) with an agitation speed of 15,000 rpm for 5 min. The nanospheres were collected from the oily suspension by centrifugation (20,000 rpm for 10 min) and rinsed with hexane (10 mL) for 5 min. The entire dispersed system was then centrifuged at 20,000 rpm for 10 min, and the sediment was suspended in 1% (w/v) PVA solution (10 mL) for 10 min. After centrifugation, an additional dispersion in water (10 mL) was carried out, and the resultant dispersion was then freeze-dried. PLGA nanospheres without glucagon were also prepared as a control. Nanosphere recovery, encapsulation efficiency and drug content were calculated using Eqs. (1), (2) and (3), respectively. The GLG/NS were dissolved in acetonitrile, to which 0.01 M HCl was added to preferentially precipitate the polymer. The drug content in the supernatant after centrifugation (14,600×*g* for 5 min) was measured using a Shimadzu class-VP HPLC system (Shimadzu, Kyoto, Japan) as reported previously (7).

$$\text{Nanosphere recovery (\%)} = \frac{\textit{weight of drug in nanospheres}}{\textit{weight of initial loading of PLGA and drug}} \times 100 \quad (1)$$

$$\text{Encapsulation efficiency (\%)} = \frac{\textit{weight of drug in nanospheres}}{\textit{weight of drug loaded in the system}} \times 100 \quad (2)$$

$$\text{Drug content (\%)} = \frac{\textit{weight of drug in nanospheres}}{\textit{weight of nanospheres recovered}} \times 100 \quad (3)$$

Respirable Powder Formulation of Glucagon

The GLG/NS-RP was prepared from jet-milled GLG/NS and carrier particles (Respitose® SV-003) as reported previously (6). Briefly, a mixture of GLG/NS (150 mg) and erythritol (150 mg) was dissolved in distilled water and lyophilized with a LyoStar II® freeze-dryer (SP Industries Inc., Warminster, PA, USA). Freeze-dried mixture was milled with an A-O JET MILL (Seishin Enterprise Co. Ltd., Tokyo, Japan). The micronized particles were mixed with a five-fold amount of carrier particles, providing GLG/NS-RP. A respirable powder formulation of glucagon without the PLGA system (GLG-RP) was also prepared for comparison. These RPs were stored in vacuum desiccators until tested. The amount of glucagon in each RP was determined by HPLC analysis.

Glucagon Fibrils

For preparation of glucagon fibrils, glucagon was dissolved in 0.01 M HCl at a concentration of 10 mg/mL, and glucagon solution was aged for 24 h at 37°C.

Electron Microscopy

For transmission electron microscopy (TEM), an aliquot (2 μL) of glucagon fibrils or water-suspended GLG/NS was placed on a carbon-coated Formvar 200 mesh nickel grid. The samples were negatively stained with 2% (w/v) uranyl acetate and visualized under an H-7600 transmission electron microscope (Hitachi, Tokyo, Japan). Representative scanning electron microscopic (SEM) images of GLG/NS-RP were taken using a VE-7800 (Keyence Corporation, Osaka, Japan).

Circular Dichroism Analysis

CD spectra of glucagon and aged glucagon dissolved in 50% MeOH/20 mM sodium phosphate buffer (NaPB) were recorded at room temperature using a Jasco model J-720 spectropolarimeter (Jasco, Tokyo, Japan) with a cell path length of 10 mm.

Cell Cultures

Rat pheochromocytoma PC12 cells, obtained from the RIKEN Cell Bank (Ibaraki, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 5% (v/v) horse serum (Gibco-BRL, Grand Island, NY, USA) and 5% (v/v) newborn calf serum (Gibco-BRL) as described previously (14). Rat alveolar L2 cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in DMEM supplemented with 10% (v/v) newborn calf serum. The cultures were maintained in 5% CO_2/95% humidified air at 37°C.

WST-8 Assay

Cells were seeded at 1×10^4 cells/well in 96-well plates (Becton Dickinson Labware, Bedford, MA, USA) at least 24 h before the experiment and cultured in serum-free DMEM supplemented with 2 μM insulin. Aged or non-aged glucagon was diluted and added to the cell culture at the indicated final concentrations. The degree of cell mortality was assayed by WST-8 conversion as reported previously (10).

Thioflavin T Binding Assay

Formation of glucagon fibril was fluorimetrically detected by ThT binding (15). Briefly, the GLG/NS was dissolved in acetonitrile, to which 0.01 M hydrochloric acid was added to preferentially precipitate the polymer. After centrifugation at 14,600×*g* for 5 min, each supernatant was diluted and adjusted to a glucagon concentration of 0.1 mg/mL. The aliquot of 20 μl of each sample was added to 1980 μl of 5 μM ThT in 20 mM phosphate-buffered saline (PBS) (pH6.0). Fluorescence was immediately measured on an RF-1500 spectrofluorophotometer (Shimadzu, Tokyo, Japan) with excitation and emission maxima of 450 and 482 nm, respectively.

Particle Size Distribution

Mean particle size of GLG/NS, suspended in 20 mM NaPB, was measured in triplicate by dynamic light scattering (DLS) using a Zetasizer Nano ZS (MALVERN, Worcestershire, UK). The particle size distribution of GLG/NS-RP was also measured by a laser diffraction scattering method using a LMS-2000e (Seishin Enterprise, Tokyo, Japan). The GLG/NS-RP were subjected to dry spraying at a pressure of 0.25 MPa for effective dispersion into fine particles and carrier molecules, and then their particle size was calculated.

Dissolution Test

Ten mg of GLG/NS were weighed into a 50 mL disposable tube and dispersed in 30 mL PBS (pH7.4). The dispersion was shaken horizontally at 37°C and 60 strokes per minute. Samples were collected at various periods and centrifuged at 14,600×*g* for 5 min. The precipitate was dissolved in acetonitrile containing 0.01 M HCl. The suspension was centrifuged at 14,600×*g* for 5 min, and the supernatant was subjected to HPLC analysis for determination of residual glucagon.

Cascade Impactor

In vitro inhalation performance on GLG/NS-RP was assessed using an AN-200 system (Shibata Science Technology, Tokyo, Japan) equipped with a JetHaler powder inhaler (Hitachi Unisia, Kanagawa, Japan) as reported previously (7,16). The GLG/NS-RP (30 mg) was weighed into a JP No.2 hard capsule of hydroxypropyl methylcellulose (Qualicaps Co. Ltd., Nara, Japan). Five capsules were subjected to cascade impactor analysis with an inspiration rate of 28.3 L/min for an inhalation time of 30 s each. The collection stages of impactor (stage 0–7) were washed with acetonitrile containing 0.01 M HCl and subjected to HPLC analysis for determination of glucagon as described in "Glucagon-Loaded PLGA Nanospheres." The fine particle dose (FPD) was defined as the mass of drug less than 5.8 μm (particles deposited at stage 2 and lower), and the fine particle fraction (FPF) was calculated as the ratio of FPD to total loaded dose.

Animals and Drug Instillation

Male Wistar rats (Japan SLC, Shizuoka, Japan), weighing 300±33 g, were housed three 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%). All procedures used in the present study were conducted in accordance with the guidelines approved by the Institutional Animal Care and Ethical Committee of the University of Shizuoka.

Rats fasted for 12 h were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and GLG/NS-RP (200 μg glucagon/kg), GLG-RP (200 μg glucagon/kg) or RP formulation of glucagon-free PLGA nanospheres (control-RP) was intratracheally administered using a powder insufflator (Model DP-4; Penn-Century, Philadelphia, PA). A bolus (2 mL) of air from an attached syringe was used to deliver the preloaded powder from the chamber of the insufflator into the airway system of the rats. The insufflator was weighed before and after powder filling as well as after administration to determine the actual dose insufflated per rat. At the appropriate time after dosing, the fasting blood glucose level and serum glucagon concentration were measured from tail-tip blood using a glucose CII-test kit (Wako, Osaka, Japan) and YK090 Glucagon EIA kit (Yanaihara, Shizuoka, Japan), respectively.

Statistical Analysis

For statistical comparisons, a one-way analysis of variance (ANOVA) with 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.

RESULTS AND DISCUSSION

Preparation of Glucagon-Loaded Nanospheres and Cytotoxic Fibril Formation

In the present study, GLG/NS were prepared by an emulsion solvent diffusion method in oil (13), in which the polymer and glucagon (1–10 mg/mL) in the emulsion droplets were co-precipitated via the diffusion of solvent. According to DLS analysis on GLG/NS re-suspended in 20 mM NaPB (pH7.4), all NS formulations exhibited unimodal particle size distribution with a mean diameter ranging from 280 to 360 nm and a polydispersity index of less than 0.8 (Table I). The recovery and drug content of the GLG/NS were variable depending on the concentration of glucagon in acetonic polymer solution. Encapsulation efficiency of GLG was found to be low, ranging from ca. 20–35%, and the highest encapsulation efficiency (35%) was observed in the GLG/NS prepared with 5 mg/mL glucagon solution. A preparation with a higher concentration of glucagon solution resulted in lower nanosphere recovery and relatively high drug content. Glucagon is easily dispersed into the water phase with mechanical agitation, and this can be part of the reasons for the limited content of glucagon in the GLG/NS prepared.

Thus, higher glucagon concentration in acetonic polymer solution is considered essential for preparing GLG/NS with sufficient drug content, possibly leading to better clinical outcomes. However, glucagon was found to be amyloidogenic in some conditions such as low pH and/or high concentration (10). In our previous study, size-exclusion chromatography analysis on glucagon aged in HCl solution suggested formation of glucagon aggregates with 2,000 kDa, whereas no marked chemical degradation of glucagon was observed (10). TEM image for a glucagon solution aged at a concentration of 10 mg/mL showed the formation of fibrils (Fig. 1A), the morphologies of which included largely disordered, rigid and branching structures stacked together edge to edge with various widths and

Table I Size and Recovery of the Glucagon-Loaded Nanospheres

	Glucagon in acetonic polymer solution		
	1 mg/mL	5 mg/mL	10 mg/mL
Particle size			
Mean diameter (nm)	350 ± 14	280 ± 44	360 ± 70
Polydispersity index	0.76 ± 0.0050	0.56 ± 0.051	0.69 ± 0.082
Recovery			
Nanosphere recovery (%)	58	42	35
Encapsulation efficiency (%)	18	35	26
Drug content (%)	0.62 ± 0.030	7.6 ± 0.030	13 ± 0.71

lengths. These resembled the cytotoxic aggregates of β-amyloid, prion and other classic amyloid fibrils (17). CD spectrum of non-aged glucagon exhibited a typical α-helical structure in 50% MeOH/20 mM NaPB, showing two negative peaks at 209 and 222 nm (Fig. 1B). In contrast, the aged glucagon displayed the typical CD spectral pattern of a ß-sheet structure with an intense positive band at ca. 200 nm and a negative extremal band at 218 nm. These observations were indicative of the formation of fibrils with abundant β-sheet structure. Previously, our group reported that aged glucagon induced apoptotic death of neuron-like cells via activation of caspase-3, an apoptotic enzyme (10). In the present study, cytotoxic effects of glucagon fibrils were assessed in rat alveolar L2 cells, as well as neuron-like PC12 cells, since the lung was the main targeted site for DPI system (Fig. 1C). Exposure of PC12 cells to the aged glucagon for 72 h resulted in a significant decrease of cell viability in a concentration-dependent manner. Interestingly, aged glucagon at concentrations over 10 μM also caused the delayed death of L2 cells as evidenced by WST-8 reduction assay and increased extracellular LDH level (data not shown). Previously, intermediates of peptide/protein fibrils, including oligomeric aggregates, were found to be more toxic to cells than mature fibrils (18). Upon these findings, if the DPI formulation of glucagon could contain cytotoxic fibrils or intermediate aggregates for some reason, glucagon-based DPI formulation might have the cytotoxic potential in pulmonary tissues.

From a quality control and safety point of view, avoidance of fibril formation during manufacturing and/or long-term storage is considered necessary for developing a glucagon-based DPI system. The structural changes of glucagon during the preparation of GLG/NS were evaluated by ThT binding assay (Fig. 2). Generally, ThT has been used to detect amyloid fibrils in pathological specimens, since it could bind specifically to amyloid-like aggregates with a ß-sheet-rich conformation. There were no significant differences in ThT binding activities between GLG/NS formulations prepared with 1 and 5 mg/mL glucagon solutions; however, GLG/NS prepared with 10 mg/mL glucagon solution exhibited ca. five-fold higher fluorescence than with 1 mg/mL. These data suggest that misfolding of glucagon caused fibril formation during preparation of GLG/NS by an emulsion solvent diffusion method in oil, and the concentration of glucagon in acetonic polymer solution should be lowered in order not to generate glucagon aggregates. In this context, the GLG/NS prepared with 5 mg/mL glucagon solution was used for further *in vitro* and *in vivo* characterization in the present study.

Sustained Release of Glucagon from Nanospheres

The GLG/NS formulation was readily re-dispersed into the aqueous medium by shaking manually, reproducing the nano-sized particles with a diameter of ca. 280 nm as determined by DLS analysis. This was in agreement with TEM images of the water-suspended GLG/NS, revealing that they were spherical and uniformly dispersed with a diameter of 200–300 nm (Fig. 3A). A smooth outer surface and a regular spherical shape could be important for extended drug release, and any irregularities would cause an increase in surface area, creating a much faster diffusive release of glucagon from the GLG/NS. Zeta potential of GLG/NS was calculated to be −41 ± 0.8 mV in water, and the negative zeta potential value could be attributed to the presence of terminal carboxylic groups in the polymers. Such a high potential value has been thought to ensure a high-energy barrier for stabilization of NS formulation (19).

Fig. 3B illustrates the *in vitro* drug release profiles for GLG/NS later used in the *in vivo* experiments, by representing the percentage of glucagon release with respect to the amount of glucagon encapsulated. Drug release occurred in two phases: a first initial burst release and a sustained release of glucagon resulting from the diffusion of the drug through the polymer matrix. As observed in other peptide-loaded NS formulations, the initial burst can most likely be attributed to glucagon adsorbed to the surface of the particles. Before optimization of the preparation process, GLG/NS was prepared with a

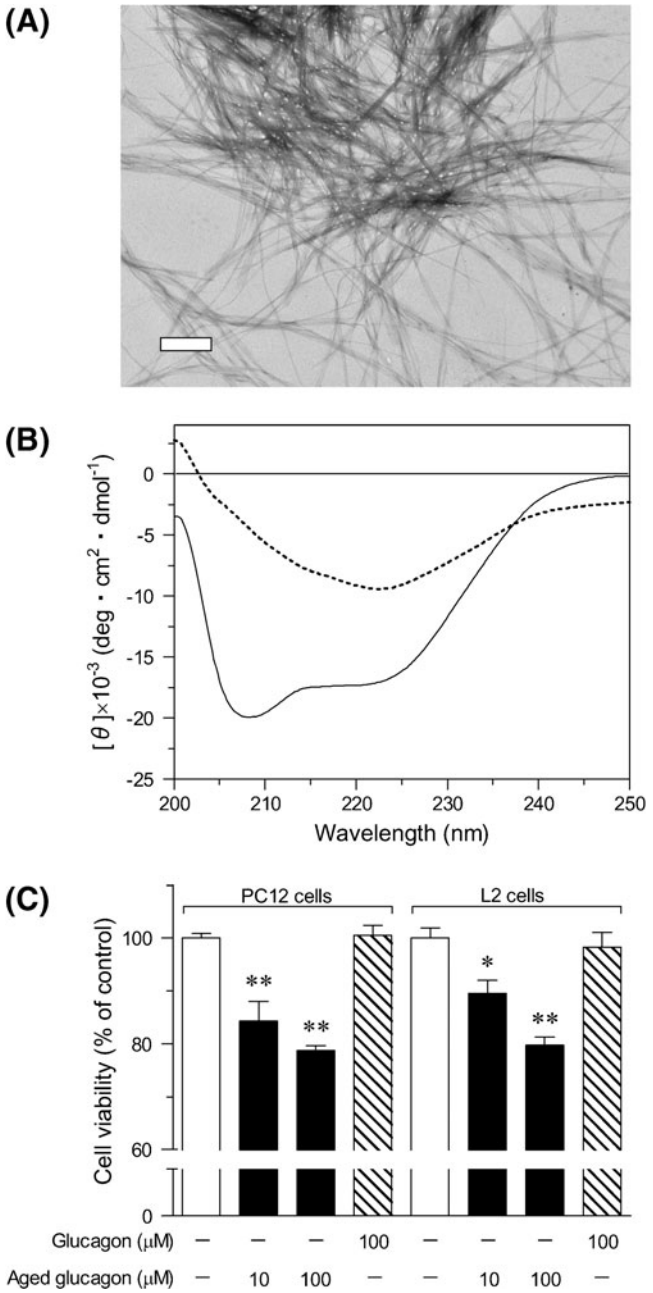

Fig. 1 Amyloidogenic properties of glucagon. (**A**) Transmission electron microscopic image of glucagon aged at 10 mg/mL in 0.01 M HCl for 24 h. Bar represents 1 μm. (**B**) CD spectra of non-aged or aged glucagon in 50% MeOH/20 mM NaPB (pH 7.4). Solid line, non-aged glucagon; dotted line, aged at 10 mg/ml. (**C**) Cytotoxicity of glucagon fibrils in neuron-like PC12 cells and rat alveolar L2 cells. Cells were treated with different concentrations of non-aged (hatched bars) or aged glucagon (filled bars) for 72 h; cell viability was determined by WST-8 assay. Data represent the mean ± SD of six determinations. **, $P<0.01$; *, $P<0.05$ with respect to the control group.

short washing time and small amount of rinse solution, there was ca. 75% burst release of total encapsulated glucagon within the first 30 min, and the drug content was calculated to be 8.7% (data not shown). The optimized preparation and washing processes could produce a 30 min burst of 32%, whereas the drug content was slightly decreased to 7.6%. In the second phase, constant slow release of glucagon up to ca. 70% of the loaded drug amount was observed within 24 h, showing a typical sustained and prolonged drug release that depends on drug diffusion and matrix erosion mechanisms (19). The sustained release behavior of glucagon from the GLG/NS might be attributable to the enhanced duration of action in inhalation therapy.

Respirable Powder Formulation of Glucagon-Loaded Nanospheres

For preparing the RP formulation of GLG/NS, a mixture of GLG/NS and erythritol was initially micronized with a jet mill. Because of their cohesive properties, micronized drugs typically have poor flow properties (20) and tend to agglomerate just after micronization. In most dry powder formulations, a carrier has been 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® with diameter of ca. 50 μm was mixed with jet-milled particles of GLG/NS and erythritol, providing GLG/NS-RP. The surface morphology of the GLG/NS-RP was visualized using SEM

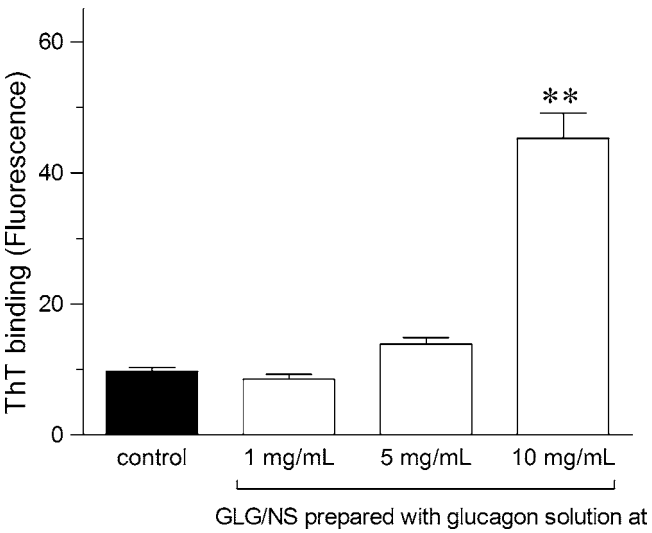

Fig. 2 Formation of glucagon fibrils during preparation of GLG/NS. The GLG/NS prepared with various concentrations of glucagon solution (1, 5 and 10 mg/mL) or non-aged glucagon (control) were dissolved in PBS (pH6.0) and diluted to glucagon concentration of 0.1 mg/mL. The glucagon fibrils in the diluted solution were detected by ThT binding assay. Data represent the mean ± SD of three determinations. **, $P<0.01$ with respect to the control.

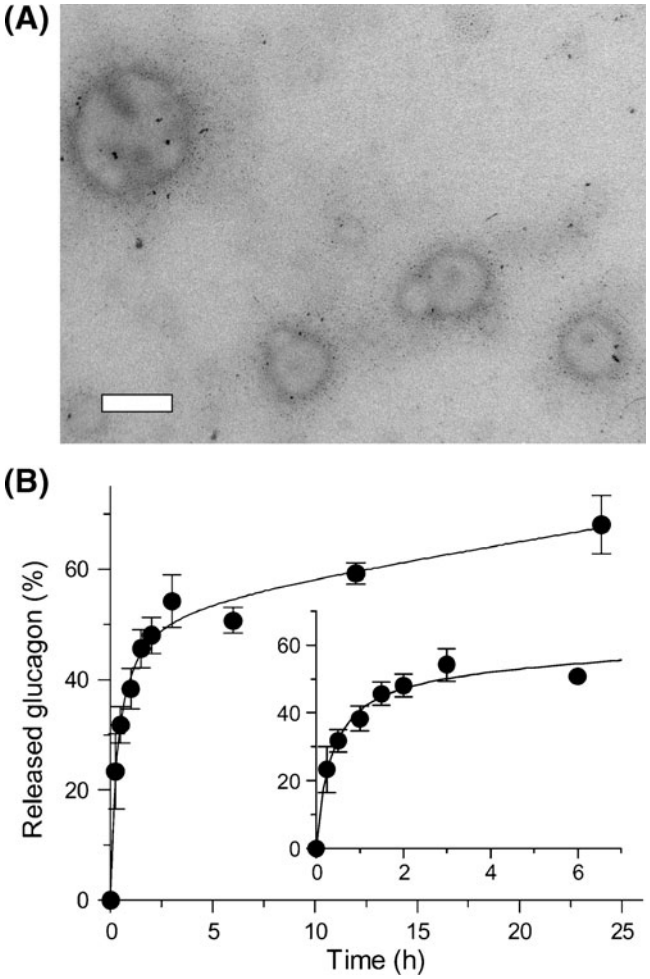

Fig. 3 Morphology and *in vitro* drug-releasing profile of GLG/NS. (**A**) Transmission electron microscopic image of GLG/NS re-dispersed in water. Bar represents 200 nm. (**B**) Cumulative *in vitro* release of glucagon from the GLG/NS in PBS. The inset highlights the early drug-releasing behavior. Data represent mean ± SE of 3 experiments.

(Fig. 4A). A SEM image from the GLG/NS-RP indicated that the micronized GLG/NS adhered to the surface of the lactose carriers. No significant agglomeration of micronized particles was observed on the surface of carrier particles, possibly due to addition of lactose carrier particles for stabilization. Without the lactose carrier, jet-milled particles seemed to be agglomerated, producing large particles (data not shown). According to the laser diffraction analysis of GLG/NS-RP (Fig. 4B), the GLG/NS-RP could be readily dispersed into a cloud of fine particles and carrier at a pressure of 0.25 MPa. There were mainly two peaks for the micronized mixture of GLG/NS and erythritol and lactose carrier, ranging from 0.2 to 13 and 13 to 120 μm, d_{50} values of which were calculated to be 5.3 and 54 μm, respectively. The micronized GLG/NS with this size diameter would be of a suitable size to avoid deposition by inertial impaction in the oropharyngeal cavity, possibly

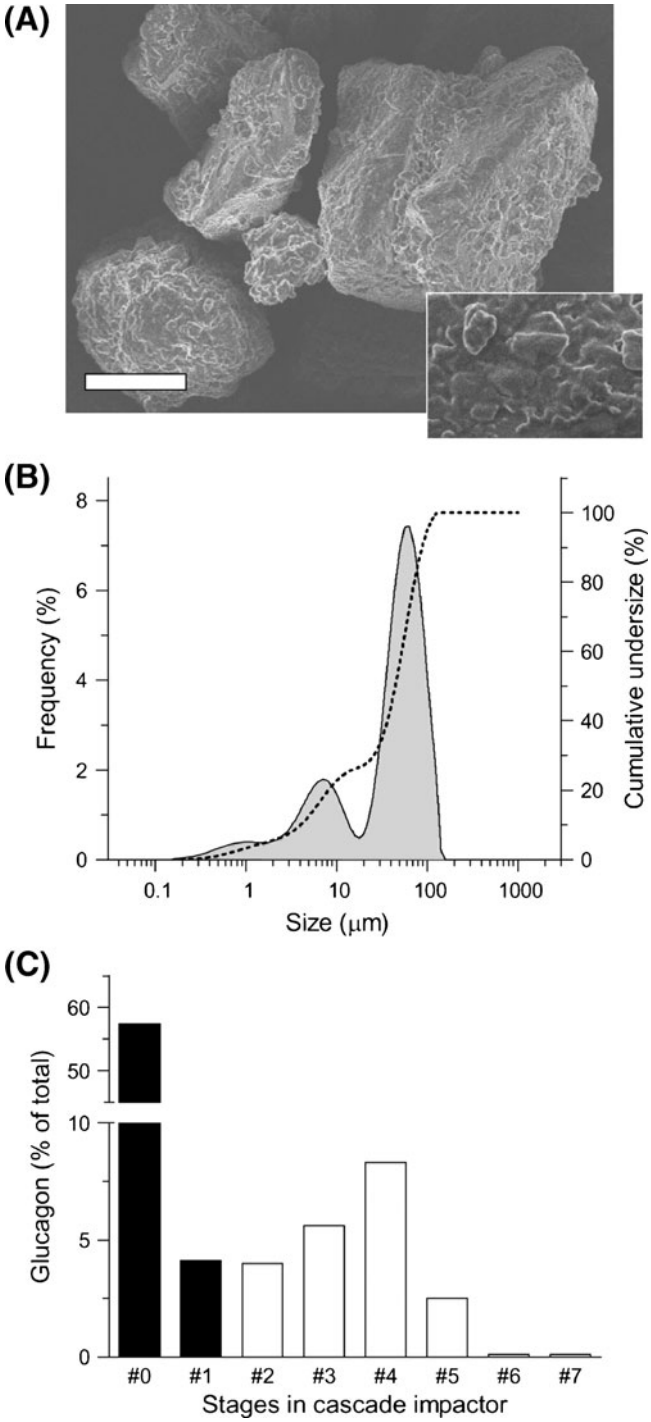

Fig. 4 Appearance and *in vitro* inhalation properties of GLG/NS-RP. (**A**) Scanning electron microscopic images of GLG/NS-RP. Bar represents 20 μm. (**B**) Particle size and aerosolization efficiency of GLG/NS-RP as determined by laser diffraction particle size analysis. The GLG/NS-RP was dispersed by dry air at a pressure of 0.25 MPa, and the size distribution and mean particle size were estimated. Solid line, frequency; dotted line, cumulative undersize fraction curve. (**C**) *In vitro* inhalation property of GLG/NS-RP. Deposition pattern analysis of GLG/NS-RP was conducted using a cascade impactor connected with a JetHaler® with an airflow rate of 28.3 L/min.

leading to effective delivery of the inhaled particles to the airway system. (21). Currently, the cascade impactor analysis is widely accepted as the standard technique for the *in vitro* characterization of aerosol clouds generated by medical aerosol generators. In the present investigation, the inhalation properties of GLG/NS-RP and its emission from the capsule were evaluated using a cascade impactor connected with JetHaler®, an inhalation device (Fig. 4C). Mass median aerodynamic diameter of the GLG/NS-RP was calculated to be 6.4 μm. On the basis of the results from cascade impactor analysis on GLG/NS-RP, the FPF value was estimated to be 21%. These findings suggested that GLG/NS-RP, employing lactose carriers, had appropriate inhalation performance, even though sticky PLGA polymer was included.

Pharmacological and Pharmacokinetic Characterization of Glucagon Formulation

Glucagon is responsible for stimulating hepatic glucose production, the primary physiological role of which, together with insulin, is the maintenance of normal glycemia (22). In the present study, glucose levels in rats were monitored after intratracheal administration of GLG-RP and GLG/NS-RP, since the transition of pharmacological responses would be indicative of enhanced duration of action due to the sustained-release system. After intratracheal administration of GLG/-RP (200 μg glucagon/kg body weight of rat as a single dose), a marked elevation of blood glucose was observed (Fig. 5A). The effect of GLG-RP reached a maximum level of 155% of the basal level at 15 min and disappeared at 90 min after intratracheal administration. Intratracheally instilled GLG/NS-RP (200 μg glucagon/kg) also exhibited potent hyperglycemic responses with the almost identical time to maximal glucose concentration as GLG-RP. According to the blood glucose level at 15 min after intratracheal administration, the GLG/NS-RP was found to be ca. 30% less effective than the GLG-RP; however, prolonged duration of action was observed in rats treated with GLG/NS-RP.

Thus, the GLG/NS-RP exhibited significant changes for the hyperglycemic effect in rats as compared with GLG-RP, suggesting that the pharmacokinetic behavior of glucagon after intratracheal administration of GLG/NS-RP might be improved as well. To verify the possible mechanisms in the enhanced duration of action, the pharmacokinetic behavior of glucagon was characterized after intratracheal administration of GLG-RP and GLG/NS-RP (Fig. 5B). According to the time evolution of serum glucagon concentration, the maximum concentration (C_{max}) of serum glucagon was calculated to be ca. 1.1 ng/mL at 15 min after intratracheal administration of GLG-RP; then the serum concentration of glucagon decreased steadily. Area under curve (AUC_{0-12}) for serum glucagon after intratracheal administration of GLG-RP was calculated to be 1,038 ± 158 pg·h/mL. While the lungs are a far less hostile metabolic environment than the gastrointestinal tract, enzymes are still present in smaller amounts (23). Glucagon, as well as other therapeutic peptides/proteins, can be enzymatically degraded intracellularly within macrophages and/or extracellularly by membrane-associated proteases and peptidases. The GLG/NS-RP also showed a T_{max} of 15 min, which partly corresponds to the initial burst release

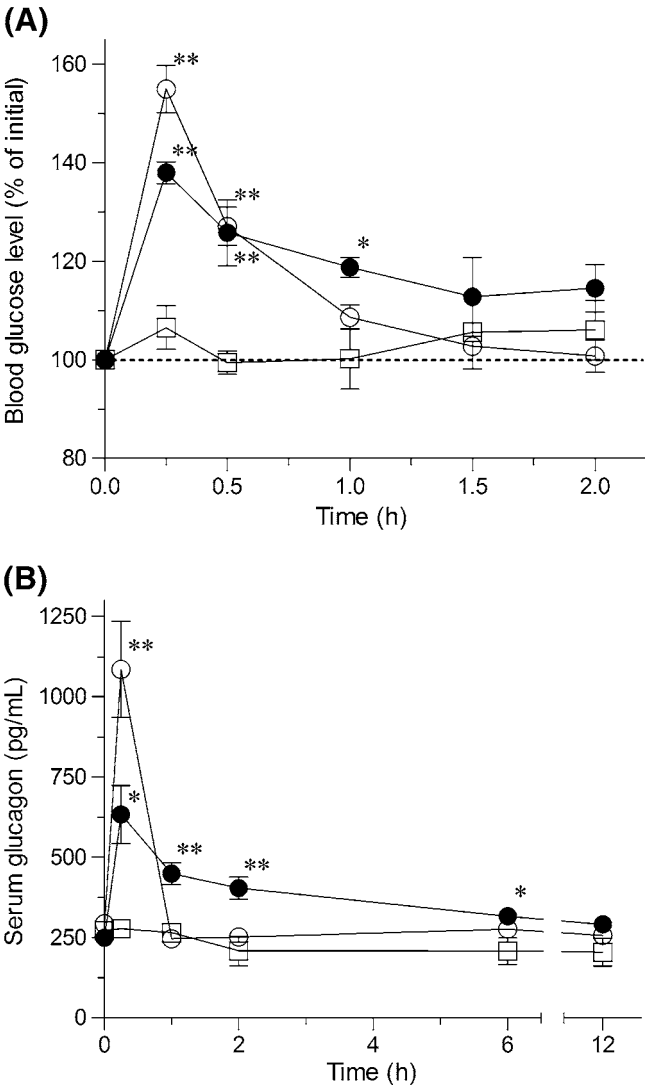

Fig. 5 *In vivo* efficacy and pharmacokinetic behavior of intratracheally instilled GLG/NS-RP in rats. (**A**) Increased level of blood glucose after intratracheal administration of glucagon formulations (200 μg glucagon/kg body weight of rat). □, control-RP; ○, GLG-RP; and ●, GLG/NS-RP. Data represent the mean ± SE of four determinations. $^{**}P < 0.01$ and $^{*}P < 0.05$ with respect to the control group. (**B**) Serum glucagon concentrations in rats after intratracheal administration of glucagon formulations (200 μg glucagon/kg body weight of rat). □, control-RP; ○, GLG-RP; and ●, GLG/NS-RP. Data represent the mean ± SE of four determinations. $^{**}P < 0.01$ and $^{*}P < 0.05$ with respect to the control group.

observed *in vitro*, whereas there appeared to be 40% decrease in C_{max} of serum glucagon compared with GLG-RP. The AUC_{0-12} value for the GLG/NS-RP was calculated to be 1,632±166 pg·h/mL. Interestingly, the elimination of serum glucagon was found to be much slower than that with the intratracheally instilled GLG-RP. There was an at least 20-fold difference in elimination rate of serum glucagon between intratracheally instilled GLG-RP and GLG/NS-RP, owing to the sustained release of glucagon from the intratracheally instilled GLG/NS-RP. These findings were in agreement with the observed pharmacological effects of intratracheally instilled GLG/NS-RP, so that the enhanced duration of action could be attributed to the improved PK behavior. According to our previous study on pharmacokinetic behavior of glucagon after intravenous administration (6), bioavailabilities of GLG-RP and GLG/NS-RP after intratracheal administration were calculated to be 0.96 and 1.52%, respectively. There were ca. 60% enhancement in bioavailability of glucagon using the sustained-release formulation approach, but it still remained low. This was consistent with previous reports (24–26), showing that physical barriers and enzymatic degradation limited bioavailability of pulmonary-delivered peptides and proteins. In this context, co-administration with enzyme inhibitors and absorption enhancers might be effective for improving the bioavailabilities and pharmacodynamic response of inhalable formulation of glucagon. Although gradual release of glucagon from GLG/NS-RP seemed to be retained even at 6 h after GLG/NS-RP challenge in rats, the hyperglycemic response disappeared within at least 1.5 h after intratracheal administration. Generally, glucagon-evoked gluconeogenesis is temporal and eventually suppressed by endogenous insulin secreted from pancreatic β-cells (27). This could partly explain the data discrepancy between hyperglycemic responses and pharmacokinetic behavior.

On the basis of the present findings, taken together with determination of glucagon fibrils in the formulation, the inhalable and sustained-release formulation of glucagon might be efficacious for maintenance of normal circulating glucose levels and improvement of metabolic status in patients with total pancreatectomy.

CONCLUSION

In the present study, inhalable long-acting formulation of glucagon was developed using a PLGA-based NS strategy. Formulation processes for the GLG/NS were optimized upon formation of cytotoxic fibrils, drug loading and formulation recovery. The GLG/NS exhibited a biphasic dissolution pattern with an initial burst and slow diffusion. According to laser diffraction and cascade impactor analyses, newly developed GLG/NS-RP, consisting of jet-milled GLG/NS and lactose carriers, had high inhalation performance with a fine particle fraction of 20.5%. *In vivo* experiments in rats demonstrated that glucagon was released in a sustained manner after intratracheal administration of the GLG/NS-RP, leading to prolonged hyperglycemic responses, compared with those with GLG-RP. These findings indicate that the new inhalable glucagon-loaded PLGA formulation could be an efficacious dosage form with improved compliance as an alternative to the injection form of glucagon currently used.

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