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Evaluation of insulin lispro and biosynthetic human insulin in pulmonary absorption: *in vivo* and *in vitro* studies

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***Objective*:** To compare the pulmonary absorption characteristics of two insulin solutions—humalog (insulin lispro) and Novolin R (Biosynthetic Human insulin) with *in vivo* and *in vitro* methods. ***Methods*:** Investigate the pharmacodynamics in Sprague Dawley (SD) rat model (*in vivo* studies) and permeability across *Rana catesbeiana* pulmonary membrane (*in vitro* studies) of Biosynthetic Human insulin (BHI) and insulin lispro (LI) at different doses. ***Results*:** Both of the insulins could reduce blood glucose levels promptly after pulmonary administration. But LI showed a better tendency on hypoglycemic effect than BHI in the *in vivo* studies. In the *in vitro* studies, the apparent permeability coefficient (Papp) for BHI and LI were almost constant with increasing concentrations, which implied that insulin maybe passively diffuse through the *Rana catesbeiana* pulmonary membrane barrier. Interestingly, the Papp of LI was obviously higher than that of BHI, indicating that the permeability of LI across *Rana catesbeiana* pulmonary membrane was more effective than that of BHI. *Conclusion*: These in vitro and *in vivo* results suggested that LI was easier to be absorbed in the lung than BHI and *Rana catesbeiana* pulmonary membrane had a potential ability, as a transport model, to predict *in vivo* pulmonary absorption of insulin.

1. Introduction

Patients with diabetes mellitus require precise and timely administration of insulin to maintain normal glycemic control. Subcutaneous injections remain the most widely used approach for insulin delivery. To overcome the problems associated with subcutaneous injection, alternative routes of insulin administration have been explored, such as the nasal, buccal, pulmonary, rectal, vaginal, conjunctival and transdermal routes (Zhang et al. 2008; Veuillez et al. 2001; Okumura et al. 1992; Onuki et al. 2000; Vermani and Garg 2000; Yang et al. 2000; Zhou et al. 2010). Among these routes, pulmonary delivery has attracted much attention because of the huge surface area of the alveolar region ($\sim$100 m^2), extensive vasculature, an ultrathinness of the alveolar epithelium (approximately 0.1–0.2 μm) and the elevated blood flow (5 l/min) (Amidi et al. 2008; Patton 1996; Hoover et al. 1992; Pilcer and Amighi 2010), which rapidly distribute molecules throughout the body. Relatively low metabolic enzyme activity in the lung makes insulin less degraded. Although the bioavailability of insulin is only about 10% after pulmonary administration without absorption enhancer (Pillion et al. 2010; Cryan et al. 2007), in humans the inhaled form of insulin is absorbed much faster (T_{max} 5–60 min) than subcutaneously injected insulin (T_{max} 60–180 min) (Chono et al. 2009). Therefore, many pulmonary administrated insulin products have been developed, tested and shown to be effective. Biosynthetic human insulin (also called regular human insulin) is often applied as material in the studies of various insulin pulmonary delivery systems, such as Sang-Ha Park's human insulin microcrystals (Park et al. 2007), Maryam Amidi's dry insulin powder (Amidi et al. 2008), and Yong Zhang's pulmonary surfactant in insulin dry powder delivery (Zhang et al. 2009; Zheng et al. 2010). Biosynthetic Human insulin is a kind of short-acting insulin made by yeast with recombinant DNA technology widely used in the treatment of diabetes. However, it could not simulate the physiological insulin secretion after subcutaneous administration and needs a careful preprandial timing to achieve near-normal postprandial glycemia. To overcome the major limitations of 'regular' human insulin, human insulin analogs have emerged (Burge et al. 1998). Insulin lispro is the first rapidly acting human insulin analog commercially available. Compared with BHI after subcutaneous injection, LI acts faster (LI's 15 min vs BHI's 15$\sim$30 min), reaches C_{max} faster (LI's 30–60 min vs BHI's 2–3 h), peaks in activity shorter (LI's 60–90 min vs BHI's 2–4 h), and has a shorter duration of action (LI's 3–5 h vs BHI's 6–8 h). Due to its superior ability to reproduce the physiological pattern of insulin secretion, better glycemic control with lower incidence of hypoglycemia, and compliance of the diabetic patients, LI has become a good choice for many patients with diabetes as supershort-effect insulin. In fact, pharmacokinetic/pharmacodynamic differences after subcutaneous administration between LI and BHI have been studied for more than a decade. However, to date little is known about the absorption difference between LI and BHI administered via the pulmonary route.

Comprehensive characterization of drug delivery to the lungs is a complex task involving the determination of delivered, deposited and absorbed dose. The pulmonary epithelial sur-

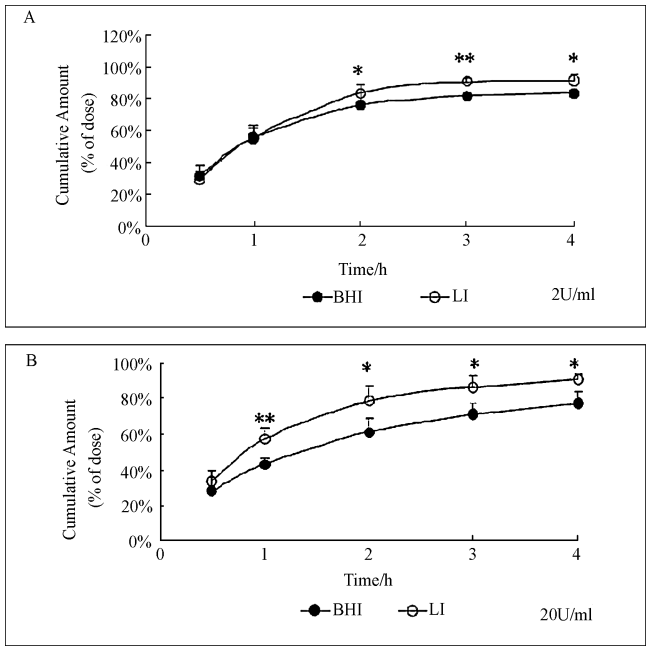

Fig. 1: Permeation profiles of BHI and LI across *Rana catesbeiana* pulmonary membrane at different doses. Each point represents the mean±SD (n = 4). BHI vs LI: $*p < 0.05$, $**p < 0.01$. A and B are for the dose of 2 U/mL and 20 U/mL, respectively.

face of mammals is relatively inaccessible, so an appropriate choice of *in vitro* model to evaluate the pulmonary absorption of insulin and elucidate their absorption mechanisms is vital during study design. However, the anatomic complexity of bronchus of mammalian lung precludes the unfolding of pulmonary tissue in diffusion chambers. In a previous report, Wall et al. (1993) developed a new method to evaluate the pulmonary transport of drugs using an amphibian lung as a model of the mammalian lung. *Rana catesbeiana* lung morphologically and physiologically resembles mammalian lung. Besides, Yamamoto et al. (2001) also applied a similar model to evaluate the permeability of insulin and the effects of absorption enhancers on its permeability. Therefore, we established an *in vitro* model using *Rana catesbeiana* pulmonary membrane to evaluate the transport characteristics and pulmonary absorption of BHI and LI.

In the present study, the pharmacodynamics in rats and permeability across *Rana* catesbeiana pulmonary membrane of BHI and LI were studied by measuring changes in blood glucose after pulmonary administration and evaluating apparent permeability coefficient (Papp) of two forms of insulin. These experiments were performed to determine if the rate and extent of pulmonary absorption of LI was similar to or different from that of BHI.

2. Investigations and results

2.1. Integrity of pulmonary membrane

Transepithelial electrical resistance (TEER) of *Rana catesbeiana* pulmonary membrane was measured during the test period. TEER was about 180 $\Omega \cdot cm^2$. There was no significant change in TEER for 4 h, confirming that the integrity of the pulmonary membrane was maintained during the transport studies.

2.2. Transport of insulin across Rana catesbeiana pulmonary membrane

Figure 1 shows the time-course of BHI and LI permeability across *Rana catesbeiana* pulmonary membrane for 4 h at different doses (low dose: 2 U/ml, high dose: 20 U/ml). The permeability of LI across the frog pulmonary membrane was faster and more effective than BHI ($p < 0.05$). Besides, the Papp values (presented in Table 1) of BHI and LI nearly did not change significantly as the concentrations increased ($p > 0.05$). Nevertheless, the permeability of LI was markedly better than that of BHI's (4.96 ± 0.17 vs 4.37 ± 0.20, 5.22 ± 0.34 vs 4.57 ± 0.19, respectively; $p < 0.05$) $\times 10^{-6}$ cm/s.

Table 1: Papp values of BHI and LI across Rana catesbeiana pulmonary membrane at different concentrations

	Dose/(U/ml)	Weight/g	Papp/($\times 10^{-6}$ cm/s)
BHI	2	220	4.37 ± 0.20
	20	156	4.57 ± 0.19
LI	2	193	4.96 ± 0.17**
	20	180	5.22 ± 0.34*

* $p < 0.05$, ** $p < 0.01$, LI compared with BHI at same dose (n = 4). The apparent permeability coefficient (Papp) was calculated from the linear portion of a plot of penetrant accumulated versus time.

2.3. Insulin absorption after intratracheal administration

In the experiment, we evaluated the relationship between the pulmonary delivered insulin dose and the hypoglycemic response (AAC). Fig. 2 presents the relation between AAC and the dose of insulin. Good relationship between the dose and efficacy parameter AAC was observed over the range of 0.2–5 U/kg. However, there was a better increase in hypoglycemic effect for LI with the increase in the dose.

The time course of glucose concentrations in blood after intrapulmonary administration of BHI and LI with three dosages is shown in Fig. 3A (5 U/kg), Fig. 3B (1 U/kg), and Fig. 3C (0.2 U/kg), and the values of pharmacodynamic parameters are shown in Table 2. The $AAC_{0\sim4}$ (the area above the blood glucose curve between zero and four hours) values for all doses of insulin tested were significantly higher than the control ($p < 0.05$) and showed a gradually increasing trend with the increase of the dose (Fig. 3). The AAC of LI performed significantly better than that of BHI at every dose (65.2 vs 56.2%·min at 0.2 U/kg, 103.4 vs 77.8%·min at 1 U/kg, 148.9 vs 106.9%·min at 5 U/kg). The t_{min} values for different doses of insulin ranged between 50 and 80 min (Table 2), but LI showed a slightly better hypoglycemic activity (D% and G_{min}%) than BHI. The period of time maintained less than 70% of initial blood glucose ($DBL_{70\%}$) is the threshold of optimal hypoglycemic effect and the '$DBL_{70\%}$' is the parameter of a long-acting property (Park et al. 2007). In this study, LI showed modest preponderance than BHI regarding the $DBL_{70\%}$ at each dose (201vs 189 min; 148 vs 115 min; 93 vs 62 min). The rate and extent of glucose reduction produced by 0.2 U/kg LI were not significantly different from those

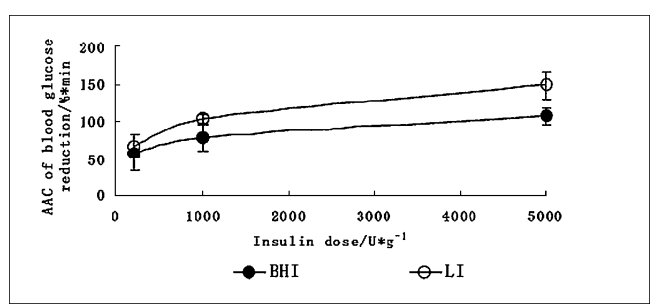

Fig. 2: Dose-hypoglycemic efficacy curve after pulmonary delivery of (●) Biosynthetic Human insulin and (○) insulin lispro. Each point presents the mean ± SD (n = 6)

Table 2: Pharmacodynamic parameters of different doses of BHI and LI after pulmonary administration

	Dose/ (U/kg)	G_{min}[a]/%	t_{min}[b]/min	AAC/(%·min)	$DBL_{70\%}$[c]/min	D%
Control	–	92.5 ± 1.3	67.5 ± 15	5.6 ± 1.3	–	–
BHI	0.2	60.2 ± 7.1	84 ± 25.0	56.2 ± 11.7	62.0 ± 29.7	22.2 ± 5.0
	1	45.0 ± 9.2	84 ± 25.1	77.8 ± 17.9	115.0 ± 31.4	31.3 ± 7.6
	5	40.4 ± 9.9	84 ± 13.4	106.9 ± 23.8	189.0 ± 30.0	43.6 ± 6.4
LI	0.2	55.8 ± 10.2*	54 ± 13.0	65.2 ± 18.5	93.0 ± 13.0	25.9 ± 7.9*
	1	33.4 ± 6.5*	65 ± 12.2	103.4 ± 5.9*	160.8 ± 42.8	42.1 ± 3.3*
	5	18.4 ± 7.0**	80 ± 15.5	148.9 ± 14.9*	201.0 ± 27.2	61.4 ± 6.4*

Control: PBS (pH 7.4). Each value represents the Mean±SD. ($n = 5$–6).
[a] G_{min}%: the percent of minimum blood glucose concentration.
[b] t_{min}: the time required to attain Gmin.
[c] $DBL_{70\%}$: the time during which less than 70% of blood glucose is held.
LI compared with BHI,* $p < 0.05$, ** $p < 0.01$.

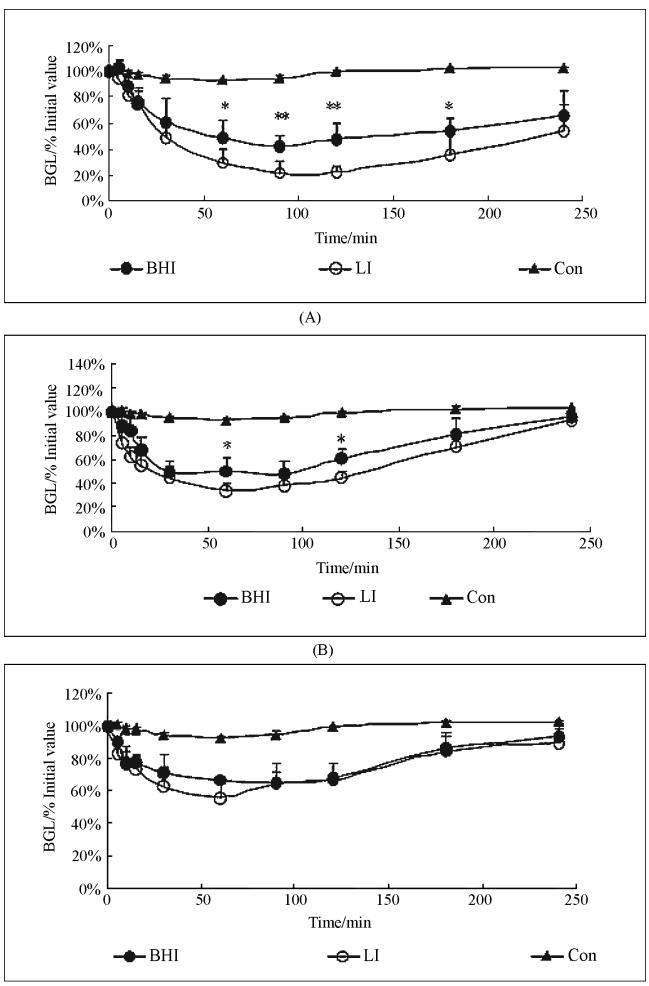

Fig. 3: Concentration-time profiles of blood glucose after pulmonary administration (i.t.) of Biosynthetic Human insulin and insulin lispro. Each point represents the mean±SD (n = 6). A, B, and C are for the dose of 5 U/kg, 1 U/kg, and 0.2 U/kg, respectively

of 0.2 U/kg BHI (Fig. 3C). On the whole, LI showed a tendency for a betters and extent of pulmonary absorption than BHI.

3. Discussion

Compared with other non-injecting route of administration, a proper *in vivo-in vitro* assessment of pulmonary administration is vital for any promising pulmonary absorption studies for peptide/protein and absorption enhancers.

Before the *in vitro* studies, the model of *Rana catesbeiana* pulmonary membrane was validated by determining transepithelial electrical resistance (TEER), comparing bilateral transport characteristic, investigating the metabolism in lung microsome of *Rana catesbeiana* pulmonary, and transporting paracellular transport standard marker (fluorescein). During the test period, TEER was kept at 180 $\Omega \cdot cm^2$, and the apparent permeability coefficients (Papp) of fluorescein was about 0.5×10^{-6} cm/s within the reported range of cell model ($0.1 \sim 0.7 \times 10^{-6}$ cm/s) (Yee 1997). The Papp of mucosal side→serosal side and serosal side→mucosal side were almost the same (4.520×10^{-6} vs 4.507×10^{-6} cm/s). The insulin did not show degradation in the lung microsome during 2 h. Therefore, the integrity, permeability of *Rana catesbeiana* pulmonary monolayer, metabolic enzymes-independent and transporter-independent permeation on the pulmonary membrane were satisfactory, which showed that the established *Rana catesbeiana* pulmonary monolayer model could be used to study the pulmonary absorption of paracellular transport drugs. Some studies had proved that proteolytic enzymes would limit the absorption of insulin in the lung (Shen et al. 1999; Morimoto et al. 2000). Shinzo Kobayashi investigated the critical factors (diffusional barrier and metabolic barrier) on pulmonary absorption of peptides and proteins, through correlation between bioavailability and molecular weight, and degradation in lung homogenates, respectively. His analytical results showed that insulin was metabolized in the lung homogenate, suggesting that the metabolic barrier was a factor in the pulmonary absorption for insulin. Therefore in our study *Rana catesbeiana* pulmonary membrane was applied successfully as a diffusional barrier, without concerning about the influence of metabolism, to investigate the transport characteristics of BHI and LI.

In vitro studies demonstrated that the permeability of insulin across the frog pulmonary membrane was good without any absorption enhancer. The Papp value of insulin across *Rana catesbeiana* pulmonary membrane was about $4.4 \sim 4.6 \times 10^{-6}$ cm/s, and this value was much higher than that of insulin across *Xenopus* pulmonary membrane (0.88×10^{-7} cm/s) (Yamamoto et al. 2001). In Yamamoto's report, the TEER of *Xenopus* pulmonary membrane was about 700 $\Omega \cdot cm^2$, and this value was higher than that of our study (180 $\Omega \cdot cm^2$). Therefore, the high permeability of insulin across the *Rana catesbeiana* pulmonary membrane was due to its low TEER value. In this study, there is little difference between 2 U/ml and 20 U/ml regarding Papp after transport studies of insulin with *Rana catesbeiana* pulmonary membrane carried out for 4 h. The influence of concentration on Papp is little, which indicates that insulin is independent of the concentration across the frog pulmonary membrane. Our result is consistent with the report of Wang and Zhang (2004), in which a human

lung adenocarcinant A549 cell line was developed to investigate the transport pathway of peptides or proteins, salmon calcitonin (sCT), insulin (INS), recombinant hirudin (rHAV2) and recombinant human growth hormone (rhGH). No concentration, no direction and temperature dependence were observed in the permeation of sCT, INS and rHAV2. The findings suggested that the hydrophilic peptides and proteins, sCT, INS, rHAV2 and rhGH appeared to penetrate the A549 cell monolayers *via* a paracellular pathway by passive diffusion mechanism. Soluble macromolecules could be absorbed from the lung into the body by two general mechanisms. One is passing through the cells (absorptive transcytosis), and the other is passing between the cells (paracellular transport). For small peptides (molecular weight < 40 kDa), paracellular transport may dominate, while for larger proteins (molecular weight > 40 kDa), transcytosis may be more important (Patton 1996). So we hypothesize that insulin (5.7 kDa) may passively diffuse through the *Rana catesbeiana* pulmonary membrane barrier by paracellular pathways. Due to the complicated transport mechanism, further studies are needed to prove this hypothesis.

In the *in vitro* permeation study, the Papp value of BHI is notably lower than that of LI (Table 1). LI showed a better permeation characteristic after adding the sampling solution to the mucosal side (Fig. 1). Usually, there is a dissociation equilibrium between polymer and monomer insulin. When monomer permeates the *Rana catesbeiana* pulmonary epithelial cells at a certain rate, the monomer could be absorbed constantly with a quicker dilution of the hexameric form of insulin in the lung and convert hexamers to monomers. LI exists in monomeric form in solution, and thus exhibits no delay in insulin permeation. While BHI tended to associate and to form dimers and/or hexamers in aqueous solutions, it was believed that hexameric insulin would dissociate into dimer or monomer before absorption, so the permeation rate of hexameric insulin may gradually increase after the initial lag. These reasons may account for the more effective transport characteristics of LI.

In the *in vivo* study, we developed intratracheal instillation to evaluate the pulmonary absorption difference between BHI and LI. The main advantages of this method are that small and relatively large doses of drug both can be delivered conveniently and that the dose delivered can be accurately measured.

In the experiment, there was a good relationship between the dose and hypoglycemic response after pulmonary administration. In initial, both insulin solutions decreased the rats' blood glucose levels rapidly, but hypoglycemic effect did not increase apparently as the dose increased, showing a "saturated phenomenon". The mechanism for the "saturated phenomenon" may be that insulin is a macromolecule, and its permeability to across pulmonary epithelial cell is little without absorption enhancers (11%∼37%). This result is similar to Alamdar Hussain's report (Hussain and Ahsan 2005). When regular hexameric and monomeric insulin of increasing doses (from 0.625 to 10 U/kg) were administered *via* the pulmonary route, there was an increase in insulin absorption with the increase in the dose, and the plasma glucose levels showed a significant reduction. But the extent of glucose reduction produced by 5 U/kg insulin was not significantly different from that produced by 10 U/kg insulin, although the amount of insulin absorbed after administration of 10 U/kg insulin was much higher than that obtained after administration of 5 U/kg insulin. The similarity in plasma glucose reduction between the two higher doses of insulin could be attributed to the lower limit of blood glucose reduction. The decrease in blood glucose values was a self-limiting factor because of the activation of counter-regulatory hormones that occurred when the blood glucose values fell very low.

After intratracheal administration of insulin solutions in rats, insulin lispro showed a tendency to a better rate and extent of pulmonary absorption than Biosynthetic Human insulin. LI produced a lower initial hypoglycemic response in comparison to that produced by BHI, and the AAC, G_{min}% and D% value of LI proved the significant differences with BHI ($p < 0.05$). Although the structure of LI is different from BHI, the difference does not significantly alter the binding of LI to the insulin receptor. In fact, LI is equipotent to human regular insulin in terms of its binding to the insulin receptor and its effects on cellular glucose uptake (Burge et al. 1998). Besides, the insulin interacts with the insulin receptor in monomeric form, so the monomeric form of LI must have a pre-receptor location, which could contribute to its superiority on the rate and extent of pulmonary absorption. Otherwise the absolute clearance rate (Cl) of LI and BHI showed no difference after intravenous injection (Holcombe et al. 2002), which could account for the reason why there were no significant differences in the duration of hypoglycemia produced by two forms of insulin. Interestingly, data on pulmonary absorption profiles of monomeric (LI) and hexameric (BHI) insulin presented in this study are similar to the studies reported by others. Liu et al. (1993) showed that the dimeric form of insulin (sodium insulin) was absorbed faster than hexameric insulin (zinc insulin) after trachea-instillation. Moreover, the duration of hypoglycemia produced by two forms of insulin was nearly the same, although dimeric form produced a lower initial hypoglycemic response in comparison to that produced by hexameric form.

In conclusion, insulin lispro may have a better tendency in pulmonary absorption than Biosynthetic Human insulin based on the *in vitro* and *in vivo* studies. Our newly developed *Rana catesbeiana* pulmonary membrane model is effective, reasonable and can be used as a screening tool to assess drug transport. It will provide a relatively complete picture for the performance of the pulmonary drug delivery device in combination with other models (Wang and Zhang 2004; Mobley and Hochhaus 2001; Sakagami 2006) concerning pulmonary drug absorption.

4. Experimental

4.1. Materials

4.1.1. Reagents

Biosynthetic human insulin injection (Novolin® R) is made by yeast with recombinant DNA technology with a content of 400U/10 ml (Novo Nordisk A/S). Recombinant human insulin lispro injection (Humalog®) is a human insulin analogue made by recombinant DNA technology with a content of 100 U/ml (Lilly France S.A.S.). The reagents for glucose assay were purchased from Shanghai Rongsheng Biotech Inc (Shanghai, China). All other chemicals were of analytical grade.

4.1.2. Animals

Male Sprague-Dawley (SD) rats, weighing 200 ± 20 g, were obtained from Zhejiang Laboratory Animal Center (Zhejiang, China). The animals were allowed *ad libitum* access to a standard diet and water. Adult African bullfrogs (*Rana catesbeiana*) of both gender weighing 150–250 g were obtained from Experimental Animal Center of China Pharmaceutical University (Nanjing, China), and kept in tap water at room temperature. All animal experiments were approved by the Animal Ethics Committee of China Pharmaceutical University and conducted in accordance with the experimental animal guidelines of China Pharmaceutical University.

4.2. Methods

4.2.1. In vitro permeation study

The permeability of insulin across the *Rana catesbeiana* pulmonary membrane was studied according to a previously reported method with little modification (Yamamoto et al. 2001). Animals were sacrificed by destroying the spinal cord with a metal probe, and the lungs were exposed, excised and placed in Ringer solution (0.65% NaCl, 0.02% $NaHCO_3$, 0.014% KCl, 0.2% Glucose, 0.001% NaH_2PO_4, and 0.012% $CaCl_2$, pH7.4). The lung

was incised, washed, unfolded, and equilibrated in Ringer solution at room temperature for 10 min. The integrity of the pulmonary membrane during the test period was monitored by measuring the transepithelial electrical resistance (TEER) with Millicell-ERS-2 (Millipore Corporations, USA).
After the equilibration period, 20 ml of Ringer solution was added to the reservoir bathing the serosal side. Sample solution (0.5 ml) was added to the mucosal side to compare the permeability of BHI and LI in different concentrations (2 U/ml and 20 U/ml). At each time point up to 4 h, 200 μl of solution was sampled from the serosal side and immediately an equal volume of buffer solution was added. These samples were analyzed by HPLC.
Data Analysis: Cumulative transmission Q_n (U), Eq. (1)

$$Q_n = V_0 \left(C_n + \frac{V}{V_0} \sum_{i=1}^{n-1} C_i \right) = V_0 C_n + V \sum_{i=1}^{n-1} C_i \quad (1)$$

in which C_n is the concentration on time t, C_i is the concentration before time t, V_0 is the volume in reservoir and V is the solution sampled from serosal side.
Apparent permeability coefficient (Papp, cm/s) estimated from the linear portion of the permeation profile was calculated by the relationship:

$$\text{Papp} = dX_R/dt \cdot 1/A \cdot C_0$$

where Papp is the apparent permeability coefficient (cm/s), X_R is the amount of drug (U), A is the diffusion area (3.4 cm^2) and C_0 is the initial concentration of drugs (U/ml) in the donor side.

4.2.2. *In vivo pulmonary administration study*

Male Sprague-Dawley (SD) rats were fasted overnight and anesthetized with sodium pentobarbital (40 mg/kg, i.p.) during the experiments. After the animal was secured on the back on an animal surgery board, the trachea was exposed and an incision was made between the fifth and sixth trachea rings caudal to the thyroid cartilage. For intratracheal delivery of drugs, a microsyringe was inserted through the incision to a depth of 12∼15 mm. Sample solution (100 μl/200 g rat) was injected directly into the trachea. Rats were maintained at an angle of 80° to horizontal for 30 s after the administration, and then at 15° during the subsequent experiments (Okumura et al. 1992; Yamamoto et al. 1992).
Rats were divided into 7 groups (6 rats in each group). One group was given phosphate buffer solution (PBS, pH 7.4) as a negative control. Three groups were given LI solution at doses of 0.2 U/kg, 1 U/kg, 5 U/kg, respectively. Three groups were given BHI at doses of 0.2 U/kg, 1 U/kg, 5 U/kg, respectively. Blood samples were withdrawn from the orbital plexus 10 min before administration and at predetermined times after dosing for up to 4 h. Serum glucose level was determined by the glucose oxidase method.
Data analysis: Blood glucose concentration was normalized by dividing the zero time glucose level (BGL/% initial value). The area above the blood glucose curve (AAC) was calculated by the trapezoidal method, Eq. (2) (Chen et al. 2002).

$$AAC_{0\sim n} = \sum_{t=0}^{n} \left\{ \left[(C_0 - C_t)/C_0 \times 100\% + (C_0 - C_{t-1})/C_0 \times 100\% \right] \times \left[t_n - t_{n-1} \right] \right\} /2 \quad (2)$$

In order to quantify the average hypoglycemic effect, D% value was calculated by the following Eq. (3) (Chono et al. 2009).

$$D\% = \frac{AUC_{PBS} - AUC_{INS}}{AUC_{PBS}} \times 100 \quad (3)$$

where AUC_{PBS} is the area under the curves of the plasma glucose levels ($AUC_{0\sim4}$) from 0 h to 4 h after administration of PBS (pH 7.4), and AUC_{INS} is the area under the curves of the plasma glucose levels ($AUC_{0\sim4}$) from 0 h to 4 h after administration of BHI or LI.

4.2.3. *Assay of insulin*

BHI and LI were separated on a Hypersil ODS2 C_{18} column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of sodium phosphate monobasic solution (adjusted to pH 3.0 with phosphoric acid) and acetonitrile (65:35). The flow rate was 1.0 mL/min and all the samples were detected at 214 nm. The standard curves of BHI and LI were As/Ais = 9.2254 C + 0.0167, R^2 = 0.9999; As/Ais = 10.105 C + 0.0791, R^2 = 0.9997, respectively. Linear calibration was generated over a concentration range of 0.01–8.0 U/ml.

4.3. *Statistical data analysis*

Results were expressed as mean ± SD. The statistical significance was determined using the Student's unpaired t-test. P values < 0.05 were considered statistically significant.

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