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

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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; Vuelles et al. 2001; Okamura 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 (∼300 m²), extensive vasculature, an ultra-thinness of the alveolar epithelium (approximately 0.1–0.2 μm) and the elevated blood flow (5 l/min) (Amidi et al. 2008; Pilcer and Amighi 2010), which rapidly distributes 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 (Tmax 5–60 min) than subcutaneously injected insulin (Tmax 60–180 min) (Chono et al. 2009). Therefore, many pulmonary administered 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 15–30 min), reaches Cmax 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-
face of mammals is relatively inaccessible, so an appropriate choice of an 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. Besides, Yamamoto et al. (2001) also applied a similar model to evaluate the permeability of mammalian lung. Therefore, we established an in vitro model using Rana catesbeiana lung morphologically and physiologically resembles mammalian lung. Besides, Yamamoto et al. (2001) also applied a similar model to evaluate the permeability of mammalian lung 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 180Ω cm². 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) cm/s.

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, the ratio of the area above the blood glucose curve between zero and four hours 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 AUC values for different doses of insulin ranged between 50 and 80 min (Table 2), but LI showed a slightly better hypoglycemic activity (p < 0.05) than BHI. The time of period maintained less than 70% of initial blood glucose (DBL 70%) was the threshold of optimal hypoglycemic effect and the DBL 70% was 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 (201 vs 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

![Figure 1: Permeation profiles of BHI and LI across Rana catesbeiana pulmonary membrane at different doses. Each point represents the mean ± SD (n = 4).](image)

![Figure 2: Dose-hypoglycemic efficacy curve after pulmonary delivery of (BHI). Biosynthetic Human insulin and (LI) insulin lispro. Each point presents the mean ± SD (n = 6).](image)

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

<table>
<thead>
<tr>
<th>Dose/(U/ml)</th>
<th>Weight/g</th>
<th>Papp/(×10⁻⁵ cm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>220</td>
<td>4.37 ± 0.20</td>
</tr>
<tr>
<td>20</td>
<td>156</td>
<td>4.57 ± 0.19</td>
</tr>
<tr>
<td>LI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>193</td>
<td>4.96 ± 0.17*</td>
</tr>
<tr>
<td>20</td>
<td>180</td>
<td>5.22 ± 0.34**</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01, LI compared with BHI at same dose (n = 6). The apparent permeability coefficient (Papp) was calculated from the linear portion of a plot of penetrant accumulated versus time.
Table 2: Pharmacodynamic parameters of different doses of BHI and LI after pulmonary administration

<table>
<thead>
<tr>
<th>Dose (U/kg)</th>
<th>Gmin (%)</th>
<th>tmin</th>
<th>AUC (%)</th>
<th>DIB, mg/lin</th>
<th>DO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>92.5 ± 1.3</td>
<td>67.5 ± 15</td>
<td>5.6 ± 1.3</td>
<td>–</td>
</tr>
<tr>
<td>0.2</td>
<td>66.2 ± 7.1</td>
<td>34.9 ± 15.4</td>
<td>106.9 ± 23.8</td>
<td>189.0 ± 30.0</td>
<td>43.6 ± 6.4</td>
</tr>
<tr>
<td>1</td>
<td>45.0 ± 9.2</td>
<td>104.8 ± 15</td>
<td>173.9 ± 31.4</td>
<td>31.3 ± 7.6</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>40.4 ± 9.9</td>
<td>84 ± 13.4</td>
<td>106.9 ± 23.8</td>
<td>189.0 ± 30.0</td>
<td>43.6 ± 6.4</td>
</tr>
<tr>
<td>LI</td>
<td>0.2</td>
<td>55.8 ± 10.2</td>
<td>54 ± 13.0</td>
<td>65.2 ± 18.5</td>
<td>93.0 ± 13.0</td>
</tr>
<tr>
<td>1</td>
<td>33.4 ± 6.5</td>
<td>65 ± 12.2</td>
<td>103.4 ± 5.9</td>
<td>160.8 ± 42.8</td>
<td>42 ± 3.3</td>
</tr>
<tr>
<td>5</td>
<td>18.4 ± 7.0</td>
<td>80 ± 15.5</td>
<td>148.9 ± 14.9</td>
<td>201.0 ± 27.2</td>
<td>61.4 ± 6.4</td>
</tr>
</tbody>
</table>

Control: PBS (pH 7.4). Each value 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. LI compared with BHI: *p < 0.05, **p < 0.01.

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 Ω·cm², and the apparent permeability coefficients (Papp) of fluorescein was about 0.5 × 10⁻⁶ cm/s within the reported range of cell model (0.1–0.7 × 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 about 4.4–4.6 × 10⁻⁶ cm/s, and this value was much higher than that of insulin across Xenopus pulmonary membrane (0.88 × 10⁻⁶ cm/s) (Yamamoto et al. 2001). In Yamamoto’s report, the TEER of Xenopus pulmonary membrane was about 700 Ω·cm², and this value was higher than that of our study (180 Ω·cm²). 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...
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 AUC, G_{max} % 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.

4. Experimental

4.1. Materials

4.1.1. Reagents

Biosynthetic human insulin injection (Novolift® R) is made by yeast with recombinant DNA technology with a content of 400 U/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 Kingsheng 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 housed in conditions of 22 ± 3°C with a 12-h light/dark cycle and had ad libitum access to a standard diet and water. Adult African bullfrogs (Rana catesbeiana) of both genders 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. A carbon dioxide/oxygen mixture was passed into the lungs, and the lungs were exposed to a temperature and pressure that were optimal for maintaining cellular integrity (100% CO2, 5% O2, and pH 7.4). The lung...
was incised, washed, unfixed, 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 transpulmonary electrical resistance (TEER) with Millicell-ERS-2 (Millipore Corporations, USA). After the equilibration period, 20% of Ringer solution was added to the reservoir subling the surgical side. Sample solution (0.5 ml) was added to the mucosal side to compare the permeability of BHI and LI in different concentrations (2U/ml and 20 U/ml). At each time point up to 4 h, 200 μl of solution was sampled from the surgical side and immediately an equal volume of buffer solution was added. These samples were analyzed by HPLC.

Data Analysis: Cumulative transmission Qc(t), Eq (1)

\[ Qc = V_0 \left( C_c - \frac{C_0}{V} \sum_{t=0}^{n} C_t \right) + \frac{V}{V_0} \sum_{t=0}^{n} C_t \]

where \( Qc \) is the concentration on time t, \( C_c \) is the concentration before time t, \( V_0 \) is the volume in reservoir and \( V \) is the solution sampled from surgical side.

Apparent permeability coefficient \( P_{app} \) (cm/s) was estimated from the linear portion of the penetration profile calculated by the relationship:

\[ P_{app} = \frac{dX}{dt} \times \frac{1}{A \times C_0} \]

\( A \) the area exposed to the mucosal side (250 μm) and \( C_0 \) is the initial concentration of drugs (μM) 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 thyroide cartilage. For intratracheal delivery of drugs, a microsyringe was inserted through the incision to a depth of 12–15 mm. Sample solution (10μl/250 g rat) was injected directly into the trachea. Rats were maintained at an angle of 80° for 10 min after administration, and then at 15° during the subsequent experiments (Okumura et al. 1992; Yamamoto et al. 1992). Rats were divided into two groups (6 rats 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. 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 (AUC) was calculated using the trapezoidal method, Eq (2) (Chen et al. 2002).

\[ AUC_{GL} = \sum_{n=0}^{n} \left[ \left( C_n - C_{n-1} \right) \times \frac{C_0}{V} \times \left( \text{time} - t_0 \right) \right] \times 2 \]

The statistical significance was determined using the Student’s unpaired t-test. P values < 0.05 were considered statistically significant.

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References


Q'app = \frac{dX}{dt} \times \frac{1}{A \times C_0} \]


