Effect of Pulmonary Surfactant and Phospholipid Hexadecanol Tyloxapol on Recombinant Human-Insulin Absorption from Intratracheally Administered Dry Powders in Diabetic Rats

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The purpose of the present study was to evaluate the enhancement effect of the natural pulmonary surfactant (PS) or its artificial substitute, phospholipid hexadecanol tyloxapol (PHT) on the bioavailability and hypoglycemic activity of recombinant human insulin (rh-insulin) in a pulmonary delivery system. PS- or PHT-loaded insulin formulation was administered to streptozotocin induced diabetic rats, at doses of 5 U/kg, 10 U/kg and 20 U/kg insulin, respectively. The hypoglycemic effect caused by PS or PHT containing rh-insulin was analyzed and the area above the curves (AUC) of serum glucose levels versus time, the minimum glucose concentration (Cmin), the time to Cmin (Tmin) and the pharmacological availability (PA%) were derived from the serum glucose profiles. Results showed that PS and PHT caused significantly decrease in serum glucose levels. The decrease in plasma glucose levels continued for about 5 h after the nadir. The highest AUC value was obtained when 20 U/kg rh-insulin with PS or PHT as absorption enhancer was administered to rats. AUC0–360min of PS- or PHT-loaded rh-insulin was 2—3 times as much as that without PS or PHT and PA% increased by 1.3—2 fold. Thus, the extent of oral absorption of insulin from PS- or PHT-loaded particles was significantly greater when compared with that without them. In addition, PHT as well as PS did not change the lactate dehydrogenase (LDH) activity, alkaline phosphatase (AKP) activity and N-acetyl-β-D-glucosaminidase (NAG) activity in bronch fluid which are sensitive indicators of acute toxicity to lung cells in bronchoalveolar lavage (BAL). It is concluded that PS and PHT is a promising absorption enhancer for pulmonary delivery systems of large molecule drugs as rh-insulin.

Key words pulmonary surfactant; phospholipid hexadecanol tyloxapol; absorption enhancer; recombinant human-insulin; lung delivery system

Pulmonary delivery of drugs has been used extensively in the treatment of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2 Recently it is also studied widely for the treatment of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2) Recently it is also studied widely for the treatment of obstructive pulmonary disease (COPD), and cystic fibrosis for many years.1,2)}
were of analytical grade.

**Animals** Male Sprague-Dawley (SD) rats weighing 170—180 g were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and housed in plastic cages in an animal holding with fixed dark and light cycle of 12 h at a constant temperature (25 ±1°C). The studies were approved by the Animal Ethics Committee of China Pharmaceutical University, and every effort was made to minimize the stress to the animals.

**Preparation of Drug Particles** The negative control and insulin-loaded dry powders were prepared by spray-drying with or without PS as described in our previous studies. Meanwhile, insulin-loaded powders with PHT as the absorption enhancer (Table 1) was prepared by the same method and the surface tension of the powders was determined. The particle sizes of PS- and PHT-loaded rh-insulin were 1.262±0.403 μm and 1.293±0.658 μm, respectively.

**Induction of Diabetes in Rats** The rats were fasted overnight before induction of diabetes. Diabetes was induced by a single intraperitoneal injection of freshly prepared streptozocin (65 mg/kg) dissolved in citrate buffer (pH 4.5). Three days after the injection of STZ, fasting blood glucose concentration was measured with a glucose oxidase-peroxidase kit (Shanghai Rongsheng Biotech Inc., Shanghai) using blood samples collected by orbital sinus puncture. Animals with blood glucose concentrations ≥16.7 mmol/l were considered diabetic and used in the following studies.

**Experimental Design** After the successful induction of diabetes, the rats were randomly divided into 11 groups each containing 5 rats, in which 9 groups received insulin dry powders with or without absorption enhancers by intratracheal administration (5, 10, 20 U/kg respectively), one group received 5 U/kg insulin by subcutaneous injection, and one group received dry powder without insulin as negative control (Table 2).

**Intratracheal and Subcutaneous Administrations of Insulin Formulations** On the day of the experiment, the animals were anesthetized by an intraperitoneal injection of pentobarbital (40 mg/ml) and secured on a board during the experiments. The trachea was exposed and a 12" needle with silicon tubing (0.9 cm in diameter) was inserted through an incision made between the fifth and sixth tracheal rings caudal to the thyroid cartilage according to the method of Enna and Schanker.11 Dry powder was put into a capsule quantitatively and then two pores were made with a needle. Insulin dry powders were dispersed in the trachea by using an aurilave synchronous with rat inspiration. After administration, the insufflator was removed and the animal was kept in an upright position for 1 min to ensure deposition of the dose. Blood samples were collected by orbital sinus puncture at 0 (immediately before administration), 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 360 min after administration. About 100 μl blood was collected in microcentrifuge tubes, placed in ice bucket until serum was separated by centrifugation (4000 rpm for 10 min). Each animal was given 1 ml of water to make up the decrease in blood volume every 1 h during the experiment.

**Bronchoalveolar Lavage** BAL was performed according to the method described by Antonietti et al.12 Five animals from each of the four exposure groups (4 mg/kg of PS, 4 mg/kg of PHT, phosphate buffer solution (PBS) as vehicle and 0.1% of sodium dodecyl sulfate (SDS) in PBS were treated 24 h before the experiment. The rats were anesthetized with sodium pentobarbital, and then exsanguinated by severing the abdominal aorta. The lungs were lavaged with two separate 3 ml aliquots of a cold, calcium and magnesium-free PBS, pH 7.4. These two BAL fluid samples were combined and centrifuged at 500 g for 10 min, and the resultant cell-free supernatant was used for the assay of LDH, NAG, and ALP activity.

**Pharmacokinetic Analysis** The hypoglycemic response to different formulations of rh-insulin was characterized as follows: serum glucose levels after insulin administrations were expressed as a percentage of the initial glucose level. The minimum percentage of serum glucose concentration (Cmin %) and the corresponding time (Tmin) were determined from the serum glucose-time profiles for all groups. The areas above the serum glucose levels time curves (AAC) were calculated using the linear trapezoidal rule.13,14 The relative pharmacological availability (PA %) of the intratracheally administered rh-insulin was calculated using the following equation.

\[
PA(\%) = \frac{AAC_{in}}{AAC_{sc}} \times \frac{Dose_{sc}}{Dose_{inh}} \times 100
\]

In which, AACin is the area above the serum glucose levels time curves of inhaled administration, AACsc is the area above the serum glucose levels time curves by subcutaneous injection, Dosein is the dose of inhaled administration, Dosesc is the dose of subcutaneous injection.

**Statistical Analysis** All results are presented as mean±S.D. Statistical differences in AAC and blood glucose levels were examined using one-way ANOVA followed by a least significant difference (LSD) post hoc test. p<0.05 was considered statistically significant.

**Results**

**Induction of Diabetic Rats** One week after STZ injection, all animals with blood glucose above 16.7 mmol/l were selected for the following experiments. The average blood glucose level was 23.65±4.73 mmol/l. The rats were randomly divided into 11 groups, and the blood glucose levels of each group are listed in Fig. 1. There was no significant difference between groups.

**The Effect of Vehicle on Blood Glucose** The influence of PHT itself on serum glucose levels was evaluated firstly. The percent reduction from the initial glucose levels versus time profiles after intratracheal administration of PHT and subcutaneous administration of 5 U/kg rh-insulin to diabetic rats were shown in Fig. 2. The mean initial serum glucose value was taken as 100% and all the following concentrations were given as percentage of the initial value. As shown in Fig. 2, subcutaneously administered insulin could significantly reduce the blood glucose. However, blood glucose of intratracheally administered vehicle did not change.

**PS and PHT on the Hypoglycemic Effect of Insulin** Figure 3 shows the changes in blood glucose levels in rats that received different doses of insulin with PS or PHT, as

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Table 1. The Formulations of rh-Insulin Dry Powders in Three Groups

<table>
<thead>
<tr>
<th></th>
<th>Negative control (mg)</th>
<th>BLANK group (mg)</th>
<th>PS group (mg)</th>
<th>PHT group (mg)</th>
</tr>
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<tbody>
<tr>
<td>Rh-insulin</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1400</td>
<td>1400</td>
<td>1200</td>
<td>1200</td>
</tr>
<tr>
<td>AAD</td>
<td>700</td>
<td>700</td>
<td>600</td>
<td>600</td>
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<tr>
<td>PS</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hexadecanol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tyloapol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18.75</td>
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</table>

Table 2. Experimental Group Details

<table>
<thead>
<tr>
<th>Dose (U/kg)</th>
<th>ins only</th>
<th>ins+PS</th>
<th>ins+PHT</th>
<th>Con.</th>
<th>S.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>5</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S.C.: subcutaneous injection

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Fig. 1. Blood Glucose Levels of Each Experimental Group after Induction of Diabetes (p>0.05)

All values were expressed as mean±S.D. No significant difference was observed.
compared to that observed in rats which received insulin alone. As shown in Fig. 3A, administration of intratracheal rh-insulin solution, at a dose of 5 U/kg, changed the plasma glucose levels slightly. However, when PS and PHT were added as absorption enhancers, blood glucose lowered further compared to that of insulin only. When the dose was increased to 10 U/kg (Fig. 3B), the hypoglycemia effect of all three groups was further enhanced. This effect lasted for 6 h, as can be seen from the blood glucose levels. The most potent blood glucose lowering effect was observed when 20 U/kg of insulin was administered (Fig. 3C). The administration of insulin alone could lower blood glucose significantly, although the blood glucose returned to 80% of initial value after 4 h. However, the blood glucose of PS and PHT group remained under 40% of initial value at the end of experiment. Compared to the blank group, the blood glucose of PS and PHT was significantly lower at each time point. Meanwhile, the glucose lowering effect of PS and PHT was comparable in all the three doses and no significant difference was observed.

The pharmacokinetic analysis was summarized in Table 3. As explained before, the equivalency of each formulation was evaluated in terms of $AAC_{0-360 min}$, $C_{min}$, $T_{min}$ and PA. Table 3 lists the $AAC_{0-360 min}$, $C_{min}$ and $T_{min}$ obtained for the

![Fig. 2. Effect of PHT on the Blood Glucose in Rats Given an Intratracheally Injection of Blank PHT as Compared to That of Subcutaneously Injected Insulin (■: Vehicle, ▲: Subcutaneously Administered Insulin)](image)

*All values were presented as mean±S.D. ∗p<0.05.

![Fig. 3. Percentage Reduction in Serum Glucose Concentration in the Diabetic Rats That Received (A), 5 U/kg, (B), 10 U/kg and (C), 20 U/kg of Insulin Respectively (●: blank, ■: PS, ▲: PHT)](image)

The rats were divided into blank insulin, PS- and PHT-loaded insulin group under each single dose. All values are expressed as mean±S.D. ∗p<0.05.

### Table 3. Parameters for Plasma Glucose Levels and Relative Pharmacological Bioavailability

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (U/kg)</th>
<th>$T_{min}$ (min)</th>
<th>$C_{min}$ (%)</th>
<th>$AAC_{0-360 min}$ (%·min)</th>
<th>PA (%)</th>
<th>PA mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.C.</td>
<td>5</td>
<td>57.5±25.8</td>
<td>16.5±4.9</td>
<td>24076.1±3370.4</td>
<td>11.45</td>
<td>10.27</td>
</tr>
<tr>
<td>ins only</td>
<td>20</td>
<td>57.0±22.2</td>
<td>38.7±14.6</td>
<td>11022.3±2544.4</td>
<td>10.55</td>
<td>8.83</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>45.8±25.8</td>
<td>65.5±14.2</td>
<td>5079.0±1321.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35.0±15.5</td>
<td>79.3±5.2</td>
<td>2125.0±598.6</td>
<td>8.83</td>
<td></td>
</tr>
<tr>
<td>ins + PS</td>
<td>20</td>
<td>62.5±24.0</td>
<td>16.8±9.5*</td>
<td>25676.3±2671.8*</td>
<td>27.08</td>
<td>26.74</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>65.0±29.5</td>
<td>37.8±9.6*</td>
<td>12754.8±2122.9*</td>
<td>26.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>55.0±27.9</td>
<td>61.9±9.6*</td>
<td>6519.1±1804.3*</td>
<td>26.66</td>
<td></td>
</tr>
<tr>
<td>ins + PHT</td>
<td>20</td>
<td>80.0±24.5</td>
<td>8.6±8.1*</td>
<td>25884.9±3214.1*</td>
<td>26.88</td>
<td>27.47</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>67.5±18.4</td>
<td>43.6±3.7*</td>
<td>13677.7±3458.4*</td>
<td>28.41</td>
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<tr>
<td></td>
<td>5</td>
<td>45.0±13.4</td>
<td>66.6±6.8*</td>
<td>6532.3±1564.5*</td>
<td>27.13</td>
<td></td>
</tr>
</tbody>
</table>

$C_{min}$, minimum serum glucose concentration (% of initial); $T_{min}$, time to $C_{min}$; $AAC$, area above the plasma glucose levels time curves; PA%, relative pharmacological bioavailability. ∗p<0.05 as compared to insulin only group of the same dose.
rats in this study. The values of $C_{\text{max}}$ and $AAC_{0-360\text{ min}}$ are significantly higher in rats that received PS or PHT as compared to those that received rh-insulin alone of the same dose. PS and PHT increased PA for 1.3—2 fold, which illustrates that PS as well as PHT, could increase the availability of insulin significantly.

**Inflammatory Parameters of Lung Injuries** The amount of NAG, LDH and ALP in the BAL fluid did not show any statistically significant increase during the exposure of PS and PHT, while the exposure of SDS increased their level significantly as compared to the unexposed group (Figs. 4A, B). The level of NAG in PHT treated group was also slightly increased when compared with the unexposed group, although not statistically significant.

**Discussion**

The lung is difficult for some drugs to permeate when formulated without the help of an absorption enhancer. Many pulmonary absorption enhancers have been tested for improving the systemic availability of macromolecular drugs from lungs. Generally speaking, these agents have been divided into following classifications, protease inhibitors, surfactants, liposomes, phospholipids and cyclodextrins. Inhaled aerosol with the size greater than 5 $\mu$m could be stopped by the oropharyngeal deposition. However, it would be directly exhaled out when it was smaller than 1 $\mu$m. Only those particles within the size range of 1 to 5 $\mu$m could reach the absorption site effectively. PS and PHT belong to the category of surfactants, and it was demonstrated that PS- and PHT-loaded rh-insulin was around 1.2 $\mu$m, an effective size to be absorbed. It is already known that PS- or PHT-loaded insulin dry powders could reduce the blood glucose in normal SD rats. This study demonstrates that addition of an absorption enhancer such as PS or PHT increases the absorption of inhaled insulin in diabetic rats.

Subcutaneously injected insulin could lower blood glucose dramatically. However, PHT, when administered alone, could not reduce the blood glucose. In rats administered all vehicle formulations, the serum glucose levels were 16% higher than the initial levels 20 min after the administration (Fig. 2). This increase is considered to be due to stress provoked by administration and blood sampling. In the whole experiment period, the blood glucose remains largely unchanged, although minor fluctuations existed. This could exclude the possible hypoglycemia effect directly from PHT.

In this study, we demonstrated for the first time that PS and PHT could enhance the pulmonary absorption of rh-insulin, whose pharmacological availability was increased from an average of 10.27 to 26.74% and 27.47% respectively. The increased availability has a direct impact on the serum glucose level, because significantly lower glycaemia was observed even at the end of the experiment period.

The hypoglycemia effect of PS was comparable to that of PHT. No significant difference of blood glucose levels between PS and PHT groups was observed. As demonstrated in Table 2, $C_{\text{max}}$ and $AAC$ values of PHT and PS were basically the same. PS and PHT could increase the pharmacological availability to about 27%, which indicates that PS and PHT have the comparable potency when used for enhancing the absorption of insulin.

Toxicological investigations are indispensable for the application of pulmonary absorption enhancers. Suzuki et al. demonstrated that high dose surfactants could induce acute lung inflammation under higher concentrations. Although it is not examined whether this effect exists under normal concentrations, this study revealed the potential toxicological effect of surfactants. In order to avoid the possible toxicity induced by injection of STZ, in the current study we adopted normal rats to examine the toxicity of the additives. NAG is a lysosomal enzyme, implying activity of phagocytic cell. LDH is a cytoplasmic enzyme, and ALP is majorly located in plasma membrane and type II cell lamellar bodies. They should be extracellular in bronchoalveolar lavage fluid only if cell lysis or cell membrane damage has occurred.

The above pulmonary damage markers in PS and PHT exposed groups didn’t increase significantly, while SDS, the positive control, increased all the parameters dramatically. This indicated a relatively low toxicity of these additives.

**Conclusion**

The results of the present study showed that PS and PHT might significantly increase the hypoglycemic effect of intratracheally administered insulin in diabetic rats. Meanwhile, PHT, as well as PS, did not change the LDH activity, ALP activity and NAG activities, which are sensitive indicators of acute toxicity to lung cells. It was concluded that PHT, which is much cheaper than its natural analogue PS, might be a promising absorption enhancer for pulmonary delivery systems of large molecule drugs as rh-insulin.

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**References**


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