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 (*AAC*) of serum glucose levels *versus* time, the minimum glucose concentration (C_{min}), the time to C_{min} (T_{min}) 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 *AAC* value was obtained when 20 U/kg rh-insulin with PS or PHT as absorption enhancer was administered to rats. $AAC_{0—360\,min}$ 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-glucoaminidase (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; asborption 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 a optional administration route of large molecules drugs because the lung has large surface area, good permeability and high blood flow.[3] Several drug delivery carriers have been used in the pulmonary administration of peptides and proteins including insulin. These can be liquid, solid or gaseous excipients, as has been reviewed by Courrier *et al.*[4] Compared with other carriers, dry powders offer more advantages, including enhanced drug stability, greater accuracy in dosing, breath-actuated delivery, improved patient compliance, and preferred delivery systems for protein and peptide drugs that are susceptible to degradation upon extended storage in aqueous solution.[5] However, under normal circumstances, the alveolar-capillary barrier prevents fast absorption of macromolecules into the blood and thus the bioavailability is not high enough to promise effective systemic therapy.[6,7] So, efficient pulmonary delivery requires drug powders with some additives (*e.g.* absorption enhancers), which can increase the amount of macromolecules reaching the blood flow. Various protease inhibitors, surfactants, lipids, polymers and other agents have been tested to improve the systemic availability of macromolecular drugs by pulmonary administration, but most of them were unsatisfactory for effectiveness or safety reasons.[8]

Pulmonary surfactant (PS), a complex mixture of 78—90% phospholipids, 5—10% proteins and 4—10% neutral lipids, is synthesized and released by type II alveolar epithelial cells mainly to reduce surface tension at the gas–liquid interface of the lung. Jing *et al.*[9] proved that artificial pulmonary surfactant had the potential to improve the bioavailability of intratracheally instilled insulin. Our previous studies revealed that PS could enhance the absorption of insulin by lung in normal rats and it might be a possible absorption enhancer.[10] However, the development of PS as absorption enhancer is difficult because of its limited content and perishable composition. Meanwhile, previous works in our laboratory showed that phospholipid hexadecanol tyloxapol (PHT), the analogical composition of PS, had similar capacity to promote the absorption of pulmonarily administered recombinant human insulin (rh-insulin) in normal rats (data not published yet). Then, it was speculated that PHT could be a promising absorption enhancer which could be applied to facilitate the pulmonary absorption of insulin.

The objective of the present study was to evaluate the *in vivo* pharmacological activity of PS- or PHT-loaded dry powders for pulmonary delivery of rh-insulin in diabetic rats. Meanwhile, the toxicity of PS and PHT was estimated through a BAL (Bronchoalveolar Lavage) method.

Experimental

Materials Streptozotocin (STZ) was obtained from Sigma-Aldrich (St. Louis, U.S.A.). Recombinant human insulin dry powders were obtained from the Department of Pharmaceutics, China Pharmaceutical University (Nanjing, China). Rh-insulin injection used in the experiments was Novolin R (regular human insulin, recombinant DNA origin Novo Nordisk, Denmark). Reagents for lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) and *N*-acetylglucosaminidase (NAG) assay kit was purchased from NanJing JianCheng Bioengineering Institute (Nanjing, China), PA Double distilled water was used for all solutions and dilution. All other reagents

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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 was prepared by spray-drying with or without PS as described in our previous studies.[10] 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 streptozococin (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 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 their backs 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 Antoniniet *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 (C_{min} %) and the corresponding time (T_{min}) 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.

$$\text{PA}(\%) = \frac{AAC_{\text{inh}}}{AAC_{\text{SC}}} \times \frac{\text{Dose}_{\text{SC}}}{\text{Dose}_{\text{inh}}} \times 100$$

In which, AAC_{inh} is the area above the serum glucose levels time curves of inhaled administration, $AAC_{SC}$is the area above the serum glucose levels time curves by subcutaneous injection, $Dose_{SC}$ is the dose of inhaled administration, $Dose_{SC}$ 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

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

	Negative control(mg)	BLANK group (mg)	PS group (mg)	PHT group (mg)
Rh-insulin	0	50	50	50
Mannitol	1400	1400	1200	1200
AAD	700	700	600	600
PS	0	0	300	0
Phospholipid	0	0	0	253.13
Hexadecanol	0	0	0	28.13
Tyloxapol	0	0	0	18.75

Table 2. Experimental Group Details

	ins only	ins+PS	ins+PHT	Con.	S.C.
5 U/kg	1	4	7	10	11
10 U/kg	2	5	8		
20 U/kg	3	6	9		

S.C.: subcutoneous injection

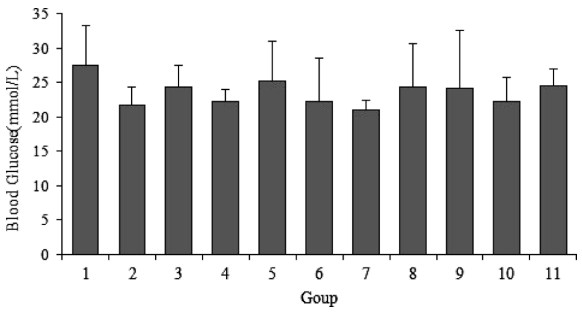

Fig. 1. Blood Glucose Levels of Each Experimental Group after Induction of Diabetes ($p>0.05$)

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.

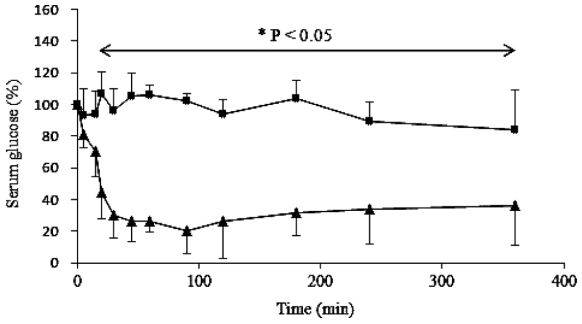

Fig. 2. Effect of PHT on the Blood Glucose in Rats Given an Intratracheally Injection of Blank PHT as Compared to That of Subcutaneously Injected Insulin (■: Vehicle, ▲: Subcutaneously Administered Insulin)

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

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

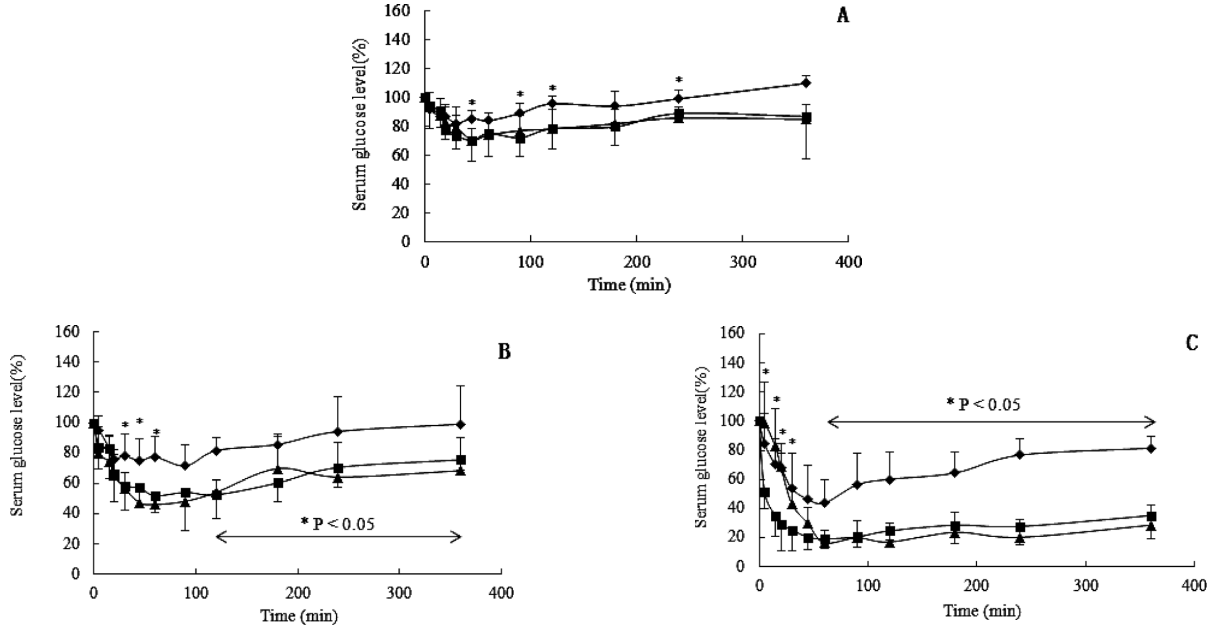

Fig. 3. Percentage Reduction in Serum Glucose Concentration in the Diabetic Rats That Received (A), 5 U/kg, (B), 10 U/kg and (C), 20 U/kg of Insulin Respectively (◆: blank, ■: PS, ▲: PHT)

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

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

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.

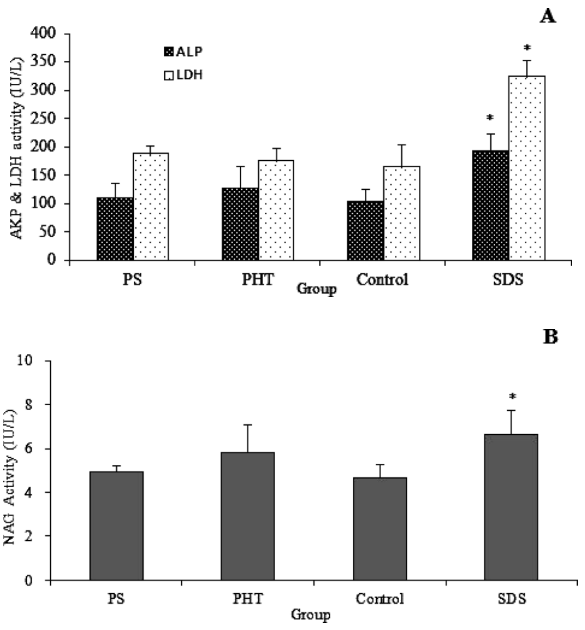

Fig. 4. Changes in Inflammatory Parameters during 24 h Exposure of PS, PHT, Control and SDS

Values were presented as mean±SD. $*p<0.05$ as compared to that of control group. No significant difference was observed between PS, PHT and control group.

rats in this study. The values of C_{max} and $AAC_{0—360\,min}$ 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.[8] 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,[15] surfactants,[16] liposomes,[17] phospholipids[18] and cyclodextrins.[19] Inhaled aerosol with the size greater than 5 μm could be stopped by the oropharyngeal deposition. However, it would be directly exhaled out when it was smaller than 1 μm. Only those particles within the size range of 1 to 5 μm could reach the absorption site effectively.[20] PS and PHT belong to the category of surfactants, and it was demonstrated that PS- and PHT-loaded rh-insulin was around 1.2 μm, 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_{min} 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.*[21] 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.[22] The above pulmonary damage markers in PS and PHT exposed groups didn't increase significantly, while SDS, the positive control,[23] 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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