Antihyperglycemic Effect of Insulin from Self-Dissolving Micropiles in Dogs

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As a percutaneous delivery device, self-dissolving micropiles (SDMPs) composed of chondroitin sulfate and insulin were prepared under room temperature from highly concentrated solution, glue. The mean weight of SDMP was 1.03±0.04 mg. One insulin SDMP was percutaneously administered to the shaved abdominal skin of four beagle dogs at insulin dose level of 1.0 and 2.0 IU/dog. After administration, blood samples were collected for 6 h and plasma glucose levels were measured. The time when minimum plasma glucose level appeared, T_{min}, was 1.38±0.2 h for 1.0 IU study and 1.38±0.1 h for 2.0 IU study and clear dose-dependent hypoglycemic effect of insulin was observed in the dose range. By comparing the area above the plasma glucose level *vs.* time curve (AAC) between insulin SDMP and subcutaneous (s.c.) injection solution, the relative pharmacological availabilities were 99% (1.0 IU) and 90% (2.0 IU), respectively. To ascertain the usefulness of insulin SDMP, oral glucose tolerance test (OGTT) was performed. When dogs were treated with insulin SDMPs, 2.0 IU, followed by an OGTT 30 min, glycemia did not appear for 5 h. On the other hand, when OGTT was performed at 1 h after insulin SDMP administration, hypoglycemia appeared as in the case of s.c. injection of insulin solution, 2.0 IU. Insulin SDMP improved the oral glucose challenge for 3 h, with a maximum effect at 30 min before the administration of glucose. Those results suggest the usefulness of a SDMP for the percutaneous delivery of peptide/protein drugs like insulin.

Key words micropile; absorption enhancement; insulin; percutaneous delivery; dog; oral glucose tolerance test

Many drug delivery systems (DDSs) including chemical enhancers, electric fields, ultrasound and thermal methods have been challenged to increase the skin permeability of poorly-absorbable drugs like insulin through the percutaneous route.[1—5] However, the success of these transdermal DDSs has been limited because of the strong barrier function of the skin *i.e.* low membrane permeability of drugs through the skin. However, micron-scale needles including micropiles made of stainless steel showed an increase of the transdermal permeability of drugs.[6—8] Among them, the permeability of insulin through the skin was dramatically increased in rats.[9] However, these microneedles are made of iron, *i.e.* stainless steel, there is a possibility of side effect like metallic allergy.

On the other hand, we have been studying a new delivery system, self-dissolving micropiles (SDMP), for the percutaneous administration of macromolecular drugs using thread-forming biopolymers such as dextrin and chondroitin sulfate. These polymers were used as a base polymer of SDMP. Dextrin was used for the base polymer of insulin SDMP in mice study.[10] However, there were some problems in fragility as a SDMP. Chondroitin sulfate could keep the viscosity in a SDMP and was selected as a base polymer because of the hardness and sharpness of the top of SDMP.[11] In our feasibility studies using mice and rats, insulin, human growth hormone (hGH) and erythropoietin (EPO) were used as representatives of peptide/protein drugs.[10,11] With insulin, the efficiency was evaluated by measuring the hypoglycemic effect of insulin after administration of insulin SDMP to mice. The pharmacological availability (PA) of SDMP was over 90% as compared to intravenously injected insulin solution.[10] Also, SDMP containing EPO and hGH showed high bioavailabilities (BA) of EPO, 80—90%, after percutaneous administration to mice and rats.[11] Through these proof-of-concept (POC) experiments, SDMP was found out to be useful for the percutaneous administration of peptide/protein drugs. Although the POC experiments were performed in small animals, the results cannot be directly applied to human, *i.e.* patients. Therefore, as a second step of the POC study, insulin SDMP was prepared and its pharmacological availability has been studied in large animal, beagle dogs, in relation to the oral glucose tolerance test (OGTT).

Experimental

Materials Insulin sodium salt was prepared from bovine pancreas insulin (25 IU/mg) in our laboratory.[12] Glucose CII-Test kit was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D-(+)-Glucose and chondroitin sulfate were obtained from Nacalai Tesque, Ltd. (Kyoto, Japan). Male beagle dogs, 10.2—12.6 kg, were obtained from Nippon SLC Co., Ltd. (Hamamatsu, Japan). All other materials used were of reagent grade and were used as received.

Preparation of Insulin Micropiles To 8.0 or 4.0 mg of sodium insulin, 60 μl of 0.1 M phosphate buffered saline (PBS, pH 7.4) was added and completely dissolved. By adding 92 mg of chondroitin sulfate to this solution, chondroitin glue containing insulin was obtained by molding well with a polypropyrene tip. The mixture was spread with the aid of polypropyrene tips and micropiles were formed, including 2 IU/mg. After the tip to which thread is attached was dried in a desiccator for 12 h, micropiles were obtained. The mean weight of the micropiles were 1.03±0.04 mg. The SDMP was conically-shaped and the mean length and basal diameter were 3.24±0.16 mm and 0.55±0.03 mm, respectively. One test preparation was contained 2.0 or 1.0 IU of insulin. Placebo SDMPs were also prepared with the same method where insulin was not added. One micropile was administered to each dog.

Preparation of Insulin Solution for Subcutaneous (s.c.) Injection Ten milliliters of PBS was added to 4.0 mg or 8.0 mg of sodium insulin and test solution, 10 IU/ml and 20 IU/ml, were prepared. 0.1 ml of each solution was injected to dogs.

***In Vivo* Absorption Experiments** Four dogs, 10.2—12.6 kg, received test insulin preparations as a cross-over manner. At 5 min before administration, blank blood sample, 0.3 ml, was obtained from the left jugular vein. After the hair of the left abdominal skin was removed, insulin SDMP was percutaneously inserted into the skin at the insulin dose of 1.0 and 2.0 IU/dog, *i.e.*, SDMP was inserted into epidermis, the thickness of which is about 100 μm. When SDMP was administered to the dog skin, the site of administration was not the dermis but the epidermis by confirming that there was no hemorrhage. At 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5 and 6 h after the ad-

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ministration, 0.3 ml of blood samples were taken from the left jugular vein. In the case of placebo SDMPs, the same procedure was used. On the other hand, insulin solution was subcutaneously injected to the right abdominal skin of the dogs after a washout period of at least one week. After collecting a blank blood sample, 0.3 ml, insulin solution was injected, 1.0 and 2.0 IU/dog.

In each sampling time, 0.3 ml of the blood sample was obtained with heparinized syringe. By centrifuging at 12000 rpm for 10 min, 100 μl of the plasma samples were obtained. All these plasma samples were immediately frozen in a deep freezer at −80 °C until analysis. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation, Kyoto Pharmaceutical University.

Glycemia after Percutaneous Administration of Insulin Micropiles Four dogs were overnight fasted and were participated to the oral glucose tolerance test (OGTT) in a cross over manner. At 30 min before the start of the experiment, blood samples, 0.3 ml, were obtained from each dog. Each dog was received unloaded (placebo) SDMP, insulin loaded SDMP and subcutaneous injection of insulin solution, respectively, with a washout period of at least one week. At 0.5 and 1.0 h after they received test preparations, glucose, solution were orally administered glucose dose of 20 g. At 0.5, 1, 1.5, 2, 3, 4 and 5 h after the administration, blood samples, 0.3 ml, were obtained from the jugular vein. By centrifuging at 12000 rpm for 10 min, 100 μl of the plasma samples were obtained. All these plasma samples were immediately frozen in a deep freezer at −80 °C until analysis.

Plasma Glucose Assay The plasma glucose level was determined using a glucose CII-Test kit. Post-dose levels of the plasma glucose were expressed as the percentage of the pre-dose level. The percentage change in the plasma glucose levels was taken as the percentage of the post-dose levels subtracted from 100. The cumulative percentage change in the plasma glucose level was calculated by summing the areas above the plasma glucose levels in the percentage change *vs.* time curves for 0—5 h (AAC) using trapezoidal method. The relative pharmacological availability (RPA) was calculated from the equation, RPA (%)=$(AAC_{percutaneous} \cdot Dose_{s.c.})/(AAC_{s.c.} \cdot Dose_{percutaneous}) \times 100$.

Statistics All values are expressed as their mean±S.E. Statistical differences were assumed to be reproducible when $p<0.05$ (Student's unpaired *t*-test).

Results

To study the linearity of the hypoglycemic effect of insulin from SDMP, insulin SDMPs were percutaneously administered to dogs at insulin dose of 1.0 and 2.0 IU/dog. Figure 1 shows the plasma glucose levels *vs.* time profiles after percutaneous administration of insulin SDMPs. As a reference, placebo SDMPs were also administered to the same dogs and the results are also shown in the figure. The minimum value of plasma glucose levels was decreased in a dose-dependent manner of insulin and the time when plasma glucose level reaches its minimum level, T_{min}, were 1.38±0.2 h for 1.0 IU study and 1.38±0.1 h for 2.0 IU study, respectively. These values were little bit greater than that obtained after s.c. injection of insulin solutions, 0.94±0.1 h for 1.0 IU and 0.88±0.2 h for 2.0 IU. The minimum plasma glucose levels, C_{min}, was represented as the percentage of the pre-dose levels was decreased in proportional to insulin dose, *i.e.* 54.5% for 1.0 IU and 33.3% for 2.0 IU studies, which were not significantly different from that observed after the s.c. injection of insulin solutions, 52.9% (1.0 IU) and 31.5% (2.0 IU). Table 1 shows the pharmacodynamic parameters of insulin SDMPs. AAC values of insulin SDMPs are 91.2%·h for 1.0 IU study and 129.2%·h for 2.0 IU study. The minimum plasma glucose level, C_{min}, was correlated to its dose. To calculate the relative pharmacological availability (RPA) of insulin, AAC values were compared between insulin SDMP and s.c. injection and then results are shown in the table. The RPAs of insulin SDMPs are 99% (1.0 IU) and 90% (2.0 IU/kg), respectively. From these results, it was suggested that percuta-

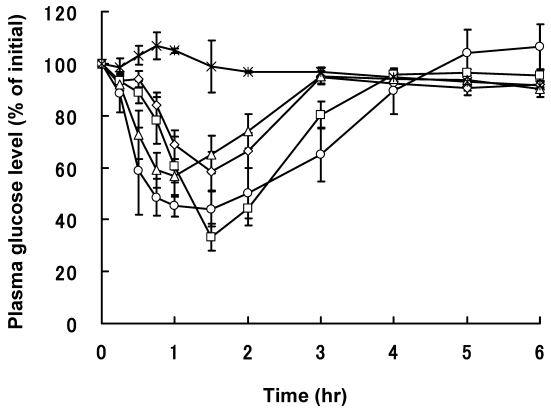

Fig. 1. Effect of Insulin Dose on Plasma Glucose Levels *vs.* Time Profiles after Percutaneous Administration of SDMP to Dogs in Comparison with s.c. Injection of Insulin

◇: insulin SDMP, 1.0 IU/dog, □: insulin SDMP, 2.0 IU/dog, ∗: placebo SDMP, △: s.c. injection of insulin solution, 1.0 IU/dog, ○: s.c. injection of insulin solution, 2.0 IU/dog.

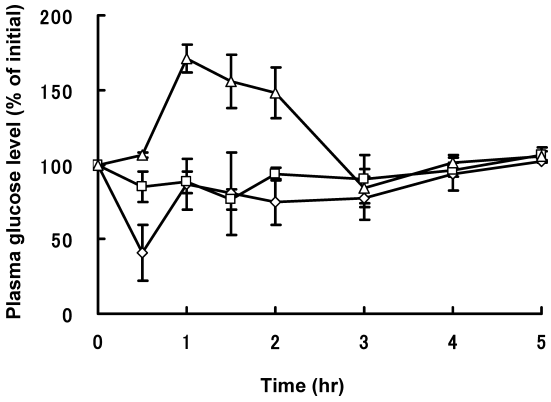

Fig. 2. Plasma Glucose Levels *vs.* Time Profiles after Percutaneous Administration of Insulin SDMP and s.c. Injection of Insulin Solution to Dogs That Received OGTT at 30 min after SDMP

□: insulin SDMP, 2.0 IU/dog, △: placebo SDMP, ◇: s.c. injection of insulin solution, 2.0 IU/dog.

Table 1. Pharmacodymanic Parameters of Insulin Percutaneously Administered with SDMPs to Dogs

Formulation	Administration route	Dose (IU/dog)	C_{min} (%)	T_{min} (h)	$AAC_{0—6}$ (%h)	RPA (%)
Solution	s.c.	1.0	52.9±4.9	0.94±0.1	94.4±10.0	100
SDMP	p.c.	1.0	54.5±5.5	1.38±0.2	91.2±3.5	99
Solution	s.c.	2.0	31.5±7.1	0.88±0.2	147.5±24.2	100
SDMP	p.c.	2.0	33.3±5.5	1.38±0.1	129.2±10.5	90

C_{min}: the minimum value of plasma glucose level. AAC: area above the plasma glucose level in the percentage change *vs.* time curve. RPA: relative pharmacological availability. Each value represents the mean±S.E. of 3—4 experiments. p.c.: percutaneous.

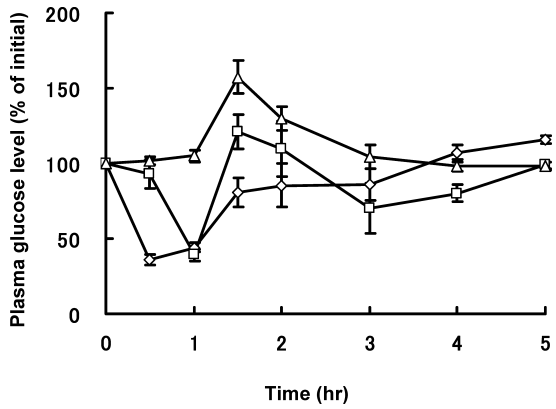

Fig. 3. Plasma Glucose Levels *vs.* Time Profiles after Percutaneous Administration of Insulin SDMP and s.c. Injection of Insulin Solution to Dogs That Received OGTT at 1 h after SDMP

□: insulin SDMP, 2.0 IU/dog, △: placebo SDMP, ◇: s.c. injection of insulin solution, 2.0 IU/dog.

neously administered insulin was well absorbed into the systemic circulation.

To ascertain the clinical usefulness of insulin SDMP, oral glucose tolerance test (OGTT) was performed and the results are shown in Figs. 2 and 3. When dogs administered with placebo SDMPs received oral glucose solution, 20 g, glycemia was increased immediately and reached to the maximum level, 171.2±9.4%, at 30 min after glucose administration. Glycemia decreased thereafter and came back to the control levels after 2.5—3.0 h. When dogs were treated with insulin SDMPs, 2.0 IU, followed by OGTT 30 min later, glycemia did not appear for 5 h. On the other hand, when insulin solution was injected subcutaneously to the dogs, 2.0 IU, hypoglycemic effect was observed at 30 min where minimum plasma glucose levels were 41.2+18.6% of the pre-dose levels. Thereafter, plasma glucose levels were returned to the normal levels. When dogs were treated with insulin SDMPs, 2.0 IU, followed by an OGTT 1 h later, hypoglycemia appeared at 1 h and minimum plasma glucose level was 39.3±4.0% of the pre-dose level. Thereafter, plasma glucose level increased to 120.7±11.4% at 1 h and came back to the normal level at 4 h. In addition, s.c. injection of insulin solution, 2.0 IU, induced a strong hypoglycemic effect for 1 h. From these results, we may state that insulin SDMP improved the oral glucose challenge for 3 h where maximum effect was obtained by administering insulin SDMP at 30 min before glucose injection.

Discussion

Clinically available insulin preparations are classified based on the access time of its action, hypoglycemia, duration of the action and the strength of the hypoglycemic activity, and have been used depending on their clinical purposes.[13,14] Among them, short-acting insulin preparation is used to prevent the hyperglycemia after each meal. On the other hand, long-acting insulin preparation is used to prevent the hyperglycemia in the daytime. Insulin SDMP belongs to the category of short-acting preparation, because hypoglycemic effect was obtained just after the administration of SDMP to the skin of mice and rats, where good dose-proportionality was observed with the hypoglycemic effect of insulin SDMPs.[10] Thus, POC study on insulin SDMP was performed with small animals. However, there is a big hurdle, species difference, between small animals and human. Therefore, insulin SDMP has been evaluated in dogs in this study. The minimum plasma glucose level, C_{min}, was also correlated to its dose. The reason why dose-proportionality was not observed with the value of AAC seemed that the individual variability appeared to be larger in dog than small animals. In this dog experiment, we designed SDMP of which one can be administered to each dog. To formulate 40 μg and 80 μg of insulin corresponding to insulin activity of 1.0 and 2.0 IU, into one the obtained SDMP, chondroitin sulfate was selected because of the hardness and sharpness of the top of SDMP. Although it was reported that the aggregation of insulin was induced by water-organic solvent interface,[15] SDMP did not induce the aggregation of insulin, including no organic solvent for preparing SDMP of chondroitin sulfate. The *in vivo* absorption experiment in dogs showed that RPA of insulin from chondroitin SDMP was above 90%. These results suggested that chondroitin sulfate was a good base for preparing insulin SDMP. With insulin, self-association is a critical factor for its activity.[16] If insulin preparation is made under high temperature, self-association would occur and dimer and hexamer are formed.[16] In this experiment, insulin SDMP was prepared under room temperature. Therefore, there is no doubt for the formation of insulin aggregates. In this study, hypoglycemic effect was obtained just after the administration of SDMP, *i.e.* 30 min, which was almost the same as to the onset time obtained after s.c. injection of insulin solution. Therefore, insulin was thought to be immediately released from SDMP as an active form after inserted into the dog skin.

The results of OGTT study also strongly support the usefulness of insulin SDMP. When SDMP was administered to dogs at 30 min before the load of glucose, glycemia was completely protected. In addition, when SDMP was administered at 1 h before glucose load, the protective effect of glycemia by insulin SDMP decreased and hyperglycemia appeared. In this case, we may state that glucose was loaded after the action of insulin SDMP started to be in the elimination phase. The most important side effects of insulin are hypoglycemia. This side effect seemed to be appeared in both conditions, *i.e.* at 30 min and 1 h before the load of glucose. On the other hand, insulin SDMP must be administered at the adequate time before each meal. From this study, it has been suggested that diabetic patients will intake insulin SDMP at 30 min before each meal.

Conclusion

The usefulness of self-dissolving micropiles (SDMPs) has been studied for the percutaneous administration of insulin in dogs. After administration of insulin SDMP, hypoglycemic effect was obtained in dogs. Dose-dependent hypoglycemic effect was observed and relative pharmacological availability (RPA) of insulin SDMP were 99% (1.0 IU) and 90% (2.0 IU), respectively. To determine the optimal time for the administration of insulin SDMP, OGTT was performed in dogs. When insulin SDMP was administered at 30 min before the administration of glucose solution, 20 g, plasma glucose levels were well maintained at the normal levels. Therefore, insulin SDMP would be clinically administered at 30 min be-

fore each meal. From those results, insulin SDMP has been shown to be a useful DDS for the percutaneous administration of insulin in diabetic patients.

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