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Improvement of Subcutaneous Bioavailability of Insulin by Sulphobutyl Ether β-Cyclodextrin in Rats

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Abstract

The objective of this study was to examine and compare how hydrophilic β-cyclodextrin derivatives (β-CyDs) improve the bioavailability of insulin following subcutaneous injection of insulin solution in rats.

When insulin solutions in the absence of β-CyDs were injected into the dorsal subcutaneous tissues of rats, the absolute bioavailability of insulin calculated from plasma immunoreactive insulin (IRI) levels was approximately 50%. When maltosyl-β-cyclodextrin was added to the solutions, there was no change in the plasma IRI levels and hypoglycaemia compared with those of the insulin-alone solution. Dimethyl-β-cyclodextrin decreased the bioavailability of insulin, although it increased the maximal concentration of IRI in plasma and the capillary permeability of the fluorescein isothiocyanate–dextran 40, a non-degraded permeation marker. When insulin solutions containing sulphobutyl ether–β-cyclodextrin with a degree of substitution of the sulphobutyl group of 3·9 (SBE4–β-CyD) were injected, the IRI level rapidly increased and maintained higher IRI levels for at least 8 h. The bioavailability of the insulin/SBE4–β-CyD system was about twice that of insulin alone and approached 96%. The enhancing effects of SBE4–β-CyD may be in part due to the inhibitory effects of SBE4–β-CyDs on the enzymatic degradation and/or the adsorption of insulin onto the subcutaneous tissue at the injection site, although this does not apparently facilitate capillary permeability.

These results suggest that SBE4–β-CyD in aqueous insulin injection for subcutaneous administration is useful for improving the bioavailability and the hence the pharmacological effects of insulin.

Diabetes mellitus is most commonly characterized by abnormal elevations of blood glucose caused by lack of insulin activity. Insulin is the mainstay for treatment of virtually all insulin-dependent diabetes mellitus (IDDM) and many non-insulin-dependent diabetes mellitus (NIDDM) patients. Recently, the optimal glycaemic control used in intensive insulin therapy was shown to delay the onset and progression of the early stages of diabetic microvascular complications in patients with NIDDM as well as in patients with IDDM (Santiago 1993).

Subcutaneous administration of insulin is the primary treatment for all patients with IDDM, and for patients with NIDDM that is not adequately controlled by diet and/or oral hypoglycemic agents. However, the bioavailability of insulin after subcutaneous administration is not high (Hori et al 1983; Kang et al 1991). Thus, attempts towards higher bioavailability and pharmacological efficacy need to be made by pharmaceutical means.

Cyclodextrins (CyDs) are known to form inclusion complexes with various guest molecules (Saenger 1980). However, the low aqueous solubility of natural CyDs, especially β-CyD, has restricted their range of applications. To improve their solubility, alkylated, hydroxyalkylated, sulphobutylated and branched CyDs have been used (Duchêne 1987; Uekama & Otagiri 1987; Szejtli 1988; Stella & Rajewski 1997). Of these hydrophilic CyDs, maltosyl–β-cyclodextrin (G_2–β-CyD), 2-hydroxypropyl–β-cyclodextrin (HP–β-CyD) and the sulphobutyl ether of β-CyD (SBE–β-CyD) have higher solubility in water and relatively

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low haemolytic activity, and thus have potential as pharmaceutical excipients for parenteral preparation (Uekama et al 1998). In fact, natural β-CyD has a toxic effect on the kidney, which is the main organ for the removal of CyDs from the systemic circulation and for concentrating CyDs in the proximal convoluted tubule after glomerular filtration (Irie & Uekama 1997). On the other hand, amorphous mixtures of highly water-soluble β-CyDs such as HP−β-CyD and SBE−β-CyDs have very low systemic toxicity compared with β-CyD. Recently, itraconazole injections containing a high HP-β-CyD content as solubilizer have been commercialized in the USA (Sporaox).

We have recently demonstrated the effects of hydrophilic β-CyDs on the aggregation of bovine insulin in aqueous solution and its adsorption onto hydrophobic surfaces. Among the CyDs tested, G_2−β-CyD potently inhibited insulin aggregation in neutral solution and its adsorption onto the surface of glass and polypropylene tubes in both concentration- and time-dependent manners, whereas dimethyl−β-cyclodextrin (DM−β-CyD) had only a moderate effect on the aggregation (Tokihiro et al 1995, 1996, 1997). In addition, SBE−β-CyD showed different effects on insulin aggregation, depending on the degree of substitution of the sulphobutyl group: SBE4−β-CyD showed inhibition at relatively low substitution levels and SBE7−β-CyD showed acceleration at high substitution levels (Tokihiro et al 1995, 1996, 1997). These results suggest that the hydrophilic β-CyDs affect the bioavailability and pharmacological effect of insulin after subcutaneous administration of insulin solution.

In this study, we mainly focused on the in-vivo absorption of insulin after subcutaneous injection of insulin in the absence and presence of hydrophilic β-CyDs (G_2−β-CyD, DM−β-CyD and SBE4−β-CyD). To gain insight into the enhancing mechanism of these β-CyDs, we compared the effects of β-CyDs on the capillary permeability of fluorescein isothiocyanate−dextran 40 (FD40), a non-degraded permeation marker, in subcutaneous tissues of rats and on in-vitro enzymatic degradation of insulin.

Materials and Methods

Materials

Bovine Zn-insulin (27·5 IU mg^{-1}; approximately 0·5% Zn) and FD40 (average molecular weight 38 260) were obtained from Sigma Chemicals (St Louis, MO). G_2-β-CyD was a gift from the Bio Research Corporation of Yokohama (Yokohama, Japan). Natural and chemically modified β-CyDs including DM−β-CyD and HP−β-CyD (average degree of substitution of 2-hydroxypropyl group = 4·0) were donated by Nihon Shokuhin Kako (Tokyo, Japan). SBE4−β-CyD (average degree of substitution of sulphobutyl group = 3·9) was donated by CyDex (Overland Park, KS) All other materials were of reagent grade, and deionized double distilled water was used.

Subcutaneous administration of insulin

Male Wistar rats, 200−250 g, were used. The phosphate-buffered saline (pH 6·8) containing insulin (0·125 mg mL^{-1}) with or without β-CyDs (25 or 100 mM) was injected subcutaneously at a dose of 2 IU kg^{-1} as insulin. Blood samples were taken periodically from the jugular veins. Plasma immunoreactive insulin (IRI) was determined by enzyme immunoassay (Glazyme Insulin-EIA Test Wako, Wako Pure Chemicals Ind., Osaka, Japan); β-CyDs did not interfere with these assays. Plasma glucose was determined by the mutarotase−glucose oxidase method (Glucose-CII-Test Wako, Wako Pure Chemicals Ind., Osaka, Japan). Plasma glucose levels after insulin administration were expressed as a percentage of the initial level. The hypoglycaemic effects of insulin preparations were expressed as the cumulative percentage of change in plasma glucose levels up to 12 h post-administration, calculated by summing the areas above (negative values) and below (positive values) the control levels obtained for the phosphate-buffered saline alone.

Permeability of FD40 through capillary

The phosphate-buffered saline (pH 6·8) containing FD40 as a non-degraded permeation marker with or without DM−β-CyD and SBE4−β-CyD (25 or 100 mM) was injected subcutaneously at a dose of 10 mg kg^{-1} as FD40, into the rats. Blood samples were taken periodically from the jugular veins. The resulting plasma (50 μL) was added to 1% (w/v) Triton X solution (1·0 μL) and FD40 in the plasma was determined using a spectrofluorimeter (Hitachi F-4010, Tokyo, Japan) at excitation 495 nm and emission 519 nm.

Stability of insulin in skin homogenates

Rats were anesthetized with urethane (1·5 g kg^{-1}) and decapitated. The skins were removed stratum corneum by tape-stripping and isolated and homogenized in a 10-fold volume of the cold phosphate-

buffered saline (pH 6·8) using a blade homogenizer (Physcotron NS-50, Nichi-On, Chiba, Japan). The homogenates were centrifuged at 10 000 *g* for 20 min at 5°C and the resulting supernatants (0·4 mL) were added to the buffer solution (0·8 mL) containing insulin (0·125 mg mL^{-1}) in the absence and presence of G_2–β-CyD (11 mM), SBE4–β-CyD (11 mM) or DM–β-CyD (2·8 mM). The mixtures were incubated at 37°C and at appropriate intervals samples (0·02 mL) of the mixture were withdrawn and added to the tube containing 0·1 N HCl solution (0·2 mL) at 0°C in order to terminate the reaction. The residual IRI in the mixture was determined by enzyme immunoassay as described above.

Statistical analysis

Data are given as the mean ± s.e.m. The statistical significance of mean comparisons was determined by an unpaired Student's *t*-test. *P* values for significance were set at 0·05.

Results and Discussion

Effects of β-CyDs on the plasma IRI level

The effects of β-CyDs on the plasma IRI level after subcutaneous injection of the phosphate-buffered saline (pH 6·8) containing insulin (2 IU kg^{-1}) to the dorsal skins of rats were examined. Figure 1 shows the plasma IRI level–time profiles after subcutaneous injection. The pharmacokinetic parameters of insulin calculated from the profiles are summarized in Table 1. The concentrations of the β-CyDs used were 100 mM, except for DM–β-CyD (25 mM), because of the relatively low 50% lethal dose value, which is 220 mg kg^{-1} in rats (Albers & Müller 1995). When the insulin-alone solution was administered, the time required to reach the maximal concentration (C_{max}) of IRI in plasma (T_{max}) was attained within about 20 min of administration and the absolute bioavailability was about 50%. This relatively large T_{max} value and lower bioavailability of insulin is in agreement with the results demonstrated by Berger et al (1982), Binder et al (1984) and Okumura et al (1985), suggesting that enzymatic and transported barriers against insulin absorption exist in the subcutaneous tissues. Indeed there are some proteases such as cathepsin-B, collagenase-like peptidase and glutathione–insulin transhydrogenase activator in subcutaneous tissues that function as enzymatic barriers (Komada et al 1985; Takayama et al 1991). In addition, the larger T_{max} value seems to indicate the existence of the transported barrier because the half-life of insulin in the rat circulation is 5–10min (Kobayashi et al 1983; Kraeger & Chisholm 1985).

To improve the pharmacokinetic behaviour of insulin after subcutaneous injection, β-CyDs were added to the solution. The addition of G_2–β-CyD affected the T_{max} value only very slightly and decreased the C_{max} value and bioavailability of insulin (Figure 1 and Table 1). DM–β-CyD also decreased the bioavailability of insulin, although it increased the C_{max} value (Figure 1 and Table 1). In addition, DM-β-CyD shortened the mean residence time (MRT) of insulin in rat plasma, although it changed the T_{max} value only very slightly (Figure 1 and Table 1). This decrease in the MRT value on adding DM–β-CyD might result from the rapidly rising and then fast lowering of plasma IRI levels, which might be due to the proteolysis of insulin at the injection sites (this is described in detail below). When insulin solutions containing SBE4–β-CyD were administered subcutaneously, the IRI level rapidly increased, the higher level was maintained for at least 8 h and the bioavailability of insulin was about twice as high as that of insulin alone with levels up to 96% (Figure 1 and Table 1). In addition, it is likely that SBE4–β-CyD increased the T_{max} and MRT values. These results indicate that the IRI level–time profiles and the pharmacokinetic parameters of insulin vary markedly depending on the type of β-CyD used, with SBE4–β-CyD appearing to have the greatest effect on raising the bioavailability of insulin in rats.

Effects of β-CyDs on capillary permeability

It is well known that β-CyDs, especially DM–β-CyD, reversibly extract phospholipids, cholesterols and proteins from biological membranes, such as erythrocytes and the nasal and intestinal epithelial cells, and decrease the integrity of the membrane, leading to an increase in the permeability of non-absorbable drugs through the membrane (Irie & Uekama 1997, 1999; Marttin et al 1998). To gain insight into the effects of SBE4–β-CyD and DM–β-CyD on the plasma IRI levels, the effects of β-CyDs on the vascular permeability at the site of injection were examined using FD40, because this compound has a molecular weight very close to that of the insulin hexamer (about 36 000 Da) and exhibits great stability under physiological conditions as well as being an non-degraded permeation marker molecule. As shown in Figure 2, DM–β-CyD resulted in a considerable enhancement of capillary permeability, as shown by the increase in the subcutaneous absorption of FD40. In sharp contrast, SBE4–β-CyD decreased the plasma levels of FD40 after subcutaneous injection, indi-

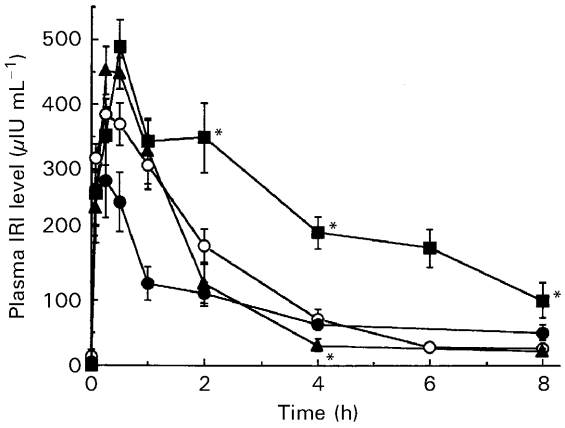

Figure 1. Effects of β-CyDs on plasma immunoreactive insulin levels after subcutaneous administration of insulin solution to rats. The concentration of insulin was 2 IU kg^{-1}. The concentrations of G_2–β-CyD and SBE4–β-CyD were 100 mM and DM–β-CyD was 25 mM. ○, Insulin alone; ●, with G_2–β-CyD; ▲, with DM–β-CyD; ■, with SBE4–β-CyD. Each point represents the mean ± s.e.m. of four to eight rats. *$P < 0.05$, compared with insulin alone.

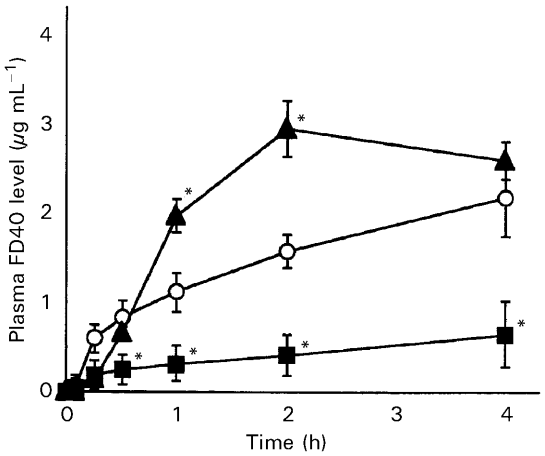

Figure 2. Effects of DM–β-CyD and SBE4–β-CyD on the subcutaneous absorption of FD40 in rats. The concentration of FD40 was 10 mg kg^{-1}. The concentrations of SBE4–β-CyD and DM–β-CyD were 100 mM and 25 mM, respectively. ○, FD40 alone; ▲, with DM–β-CyD; ■, with SBE4–β-CyD. Each point represents the mean ± s.e.m. of three rats. *$P < 0.05$, compared with FD40 alone.

cating that SBE4–β-CyD inhibited the permeability of FD40 through capillaries. The enhancing effect of DM–β-CyD on the capillary permeability may therefore lead to an increase in the C_{max} value of plasma IRI, as shown in Table 1. However, DM–β-CyD somewhat decreased the bioavailability of insulin. This might be explained as follows. DM–β-CyD is rapidly absorbed along with insulin from the injection site into the bloodstream in terms of its enhancing effect on the capillary permeability, followed by a decrease in the effective concentration of DM–β-CyD required for the protective effect on the proteolysis of insulin. This may lead to a decrease in the bioavailability as well as the MRT value, with a rapid lowering of the plasma IRI levels as described above. On the other hand, it was suggested that SBE4–β-CyD improves insulin bioavailability by mechanisms other than capillary permeability.

Protective effects of β-CyDs on insulin proteolysis

It is well known that hydrophilic β-CyDs stabilize proteins and peptides such as α-chymotrypsin and buserelin acetate against degradation via proteases (Matsubara et al 1995, 1997). Figure 3 shows the effects of β-CyDs on insulin degradation in rat skin homogenates. In this study it was decided to use identical ratios of insulin/β-CyDs in both in-vivo and in-vitro solutions in order to make a more useful comparison. The IRI levels in homogenates rapidly decreased in the absence of β-CyDs, suggesting the proteolysis of insulin in the homogenates. On the other hand, β-CyDs tended to retard the decrease in IRI levels, with SBE4–β-CyD having the largest inhibitory effect. Table 2 depicts the first-order degradation rate constants (k) of insulin and the half-lives ($t_{1/2}$) in the absence and presence of β-CyDs. The k values were reduced by adding β-CyDs in the order: insulin alone > G_2–β-CyD > DM–β-CyD > SBE4–β-CyD. Reflecting the k values, the $t_{1/2}$ values were increased by adding β-CyDs, for example DM–β-CyD and SBE4–β-CyD increased about 1·2- and 1·4-fold over the control value, respectively. This stabilizing effect of DM–β-CyD on the proteolysis of insulin is unlikely to be consistent with the results for the

Table 1. Systemic bioavailability of insulin following subcutaneous administration of insulin solution (2 IU kg^{-1}) with or without G_2–β-CyD (100 mM), DM–β-CyD (25 mM) and SBE4–β-CyD (100 mM) in rats.

System	C_{max} (μIU mL^{-1})	T_{max} (h)	MRT (h)	AUC (μIU mL^{-1} h^{-1})	F (%)
Insulin alone	396 ± 25	0·3 ± 0·1	1·7 ± 0·1	700 ± 92	50 ± 6
With G_2–β-CyD	304 ± 62	0·3 ± 0·1	2·0 ± 0·1	449 ± 61	32 ± 4
With DM–β-CyD	517 ± 34*	0·4 ± 0·1	1·3 ± 0·1*	610 ± 102	44 ± 7
With SBE4–β-CyD	492 ± 46	0·8 ± 0·4	2·5 ± 0·3	1336 ± 248*	96 ± 14*

Each value represents the mean ± s.e.m. of three to eight rats. AUC calculated up to 8 h post-administration. F = Bioavailability compared with the AUC value of insulin administered intravenously (0·4 IU kg^{-1}). *$P < 0.05$ compared with insulin alone.

decrease in the bioavailability and MRT value of insulin (see Table 1). This inconsistency may be due to the difference in the experimental conditions: the relatively low stabilizing effect of DM–β-CyD on the insulin proteolysis and competitive inclusion, i.e. the dissociation of insulin from the complex with DM–β-CyD by some competing molecules (phospholipids and cholesterols) in the biological membranes, such as the capillary membrane under the in-vivo conditions. On the other hand, this stabilizing effect of SBE4–β-CyD on the insulin degradation in the homogenates could be attributed to both direct and indirect inhibitory effects, i.e. the direct effect means that SBE4–β-CyD may protect insulin from enzymatic attack via complexation with insulin on the basis of the relatively high intermolecular interaction (Tokihiro et al 1997), whereas the indirect effect means that SBE4–β-CyD may decrease the enzymatic activity of proteases, as reported previously for the α-chymotrypsin and buserelin acetate systems (Matsubara et al 1995, 1997). However, SBE4–β-CyD inhibited the capillary permeability of FD40 (see Figure 2). This suggests that SBE4–β-CyD was allowed to retain insulin at the injection site and eventually was subject to proteolysis of insulin there. Taking these effects of SBE4–β-CyD on the proteolysis and the capillary permeability into consideration, the increase in the plasma IRI levels under the present in-vivo conditions may be explained by the idea that the protective effects of SBE4–β-CyD against the proteolysis surpassed the negative effect, which retains insulin in the subcutaneous tissue where there are a lot of proteases. However, other factors should be considered because the protective effects of β-CyDs, even for SBE4–β-CyD, were not sufficient to provide a complete explanation.

Effects of β-CyDs on hypoglycemia

Figure 4 shows the plasma glucose level–time profiles after subcutaneous administration of the phosphate-buffered saline containing insulin in the absence and presence of β-CyDs. When insulin-alone solution was injected, the minimal levels of glucose occurred 4 h after injection and the plasma glucose levels then gradually recovered to basal levels. A less enhancing effect of G_2–β-CyD on the hypoglycaemia after insulin was observed. In the case of the DM–β-CyD system, the maximum hypoglycaemic effect occurred 2 h after administration, and the glucose levels then rapidly recovered. On the other hand, SBE4–β-CyD provided the greatest hypoglycaemic effect, which was maintained for up to 8 h. Thus, it is likely that these

Table 2. First-order rate constants (k) and half-lives ($t_{\frac{1}{2}}$) of insulin degradation in rat skin homogenates at 37°C.

System	k ($\times 10^{-2} h^{-1}$)	$t_{1/2}$ (h)
Insulin alone	7·73 ± 0·62	9·26 ± 0·74
With G_2–β-CyD	6·24 ± 0·60	11·64 ± 1·12
With DM–β-CyD	6·10 ± 0·09*	11·38 ± 0·17*
With SBE4–β-CyD	5·54 ± 0·28**	12·66 ± 0·61**

Each value represents the mean ± s.e.m. of six to nine experiments. *$P < 0·05$, **$P < 0·01$ compared with insulin alone.

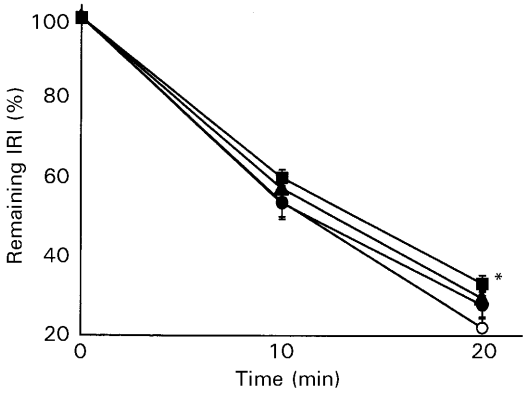

Figure 3. Effects of β-CyDs on the degradation of insulin in rat skin homogenates at 37°C. The concentrations of G_2–β-CyD, DM–β-CyD and SBE4–β-CyD were 11 mM, 2·8 mM and 11 mM, respectively. ○, Insulin alone; ●, with G_2–β-CyD; ▲, with DM–β-CyD; ■, with SBE4–β-CyD. Each point represents the mean ± s.e.m. of six to nine experiments. *$P < 0·05$, compared with insulin alone.

effects of β-CyDs on the hypoglycemia reflected the plasma IRI level profiles (Figure 1). Here β-CyDs alone did not lead to any changes in plasma levels of endogenous insulin and glucose levels (data not shown).

As described in our previous papers (Tokihiro et al 1995, 1996, 1997), G_2–β-CyD markedly inhibited the aggregation and adsorption of insulin among the β-CyDs used here. Thus, we expected insulin solutions containing G_2–β-CyD to be rapid-acting insulin preparations with greater bioavailability and hypoglycaemic effects. However, no enhancing effect of G_2–β-CyD on bioavailability and hypoglycaemia was observed (see Figure 1). This may be explained as follows. G_2–β-CyD may dissociate the dimer or hexamer of insulin, leading to an increase in the susceptibility of insulin to the peptidases, and/or the relatively high concentration of G_2–β-CyD may increase the viscosity of the solution (Yamamoto et al 1989), leading to a decrease in the diffusibility of insulin. Also, the lack of enhancing effects of DM–β-CyD on insulin bioavailability and hypoglycaemia can be explained by similar mechanisms as for G_2–β-CyD

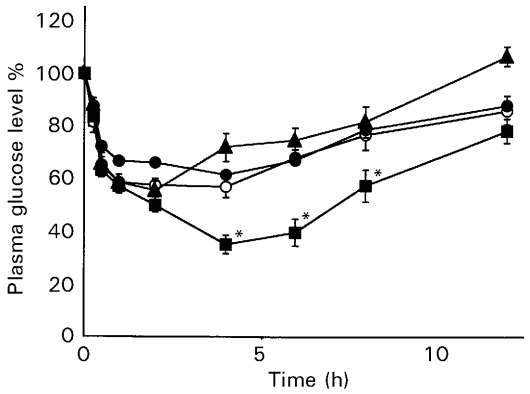

Figure 4. Plasma levels of glucose after subcutaneous administration of insulin solution with or without β-CyDs to rats. The concentration of insulin was 2 IU kg^{-1}. The concentrations of $G_2-\beta$-CyD and SBE4$-\beta$-CyD were 100 mM and that of DM$-\beta$-CyD was 25 mM. The AUC represents the cumulative percentage of change in plasma glucose levels up to 12 h post-administration. ○, Insulin alone; ●, with $G_2-\beta$-CyD; ▲, with DM$-\beta$-CyD; ■, with SBE4$-\beta$-CyD. Each point represents the mean ± s.e.m. of at least four rats. *$P < 0.05$, compared with insulin alone.

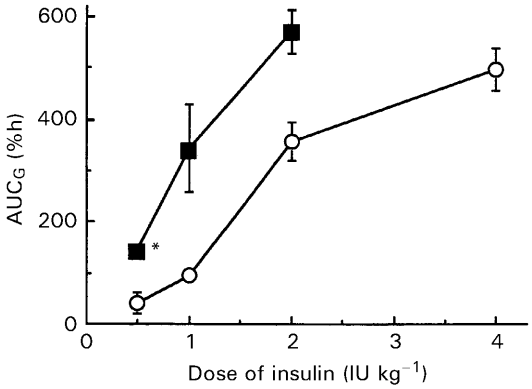

Figure 5. Dose-dependence of the hypoglycaemic effect of insulin solution with or without SBE4$-\beta$-CyD after subcutaneous administration to rats. The dose of insulin and the concentration of SBE4$-\beta$-CyD were 0·5–4 IU kg^{-1} and 100 mM, respectively. ○, Insulin alone; ■, with SBE4$-\beta$-CyD. Each point represents the mean ± s.e.m. of four to eight rats. *$P < 0.05$, compared with insulin alone.

because they are both non-ionized hydrophilic β-CyDs, although there are some differences in the effects on capillary permeability and insulin stability, as described above. On the other hand, SBE4$-\beta$-CyD, an anionic β-CyD derivative, enhanced both the bioavailability and hypoglycaemic effect of insulin. We previously revealed that SBE4$-\beta$-CyD interacts with insulin in a different manner to $G_2-\beta$-CyD and DM$-\beta$-CyD, i.e. SBE4$-\beta$-CyD greatly interacts with the insulin molecule in terms of not only the intermolecular forces such as the hydrophobic bond, hydrogen bond and van der Waals force but also the electrostatic force with basic amino acids (Arg^{22}, His^{5,10} and Lys^{29} in the B-chain of insulin) (Tokihiro et al 1997). The relatively strong electrostatic interaction of insulin with SBE4$-\beta$-CyD as well as the inclusion complexation may therefore be of importance for the improvement in the potent bioavailability and hypoglycaemic effect of insulin after subcutaneous injection.

The dose-dependent effect of insulin on hypoglycaemia after subcutaneous injection was also examined. The *x*- and *y*-axes in Figure 5 indicate the insulin dose and the area under the plasma glucose level–time curves (AUC) up to 12 h post-administration, respectively. When the insulin-alone solution was administered, the AUC values markedly decreased with decreasing dose in a non-linear manner. On the other hand, in the SBE4$-\beta$-CyD system, the AUC value was almost proportional to the insulin dose as compared with that of insulin alone. This non-linear pattern observed in the lower insulin dose region could be attributed to the proteolysis of insulin caused by the dissociation of the dimer and hexamer of insulin and/or the adsorption of insulin in the subcutaneous tissue in the injection site. The enhancing effects of SBE4$-\beta$-CyD may therefore be due in part to the inhibitory effects of SBE4$-\beta$-CyD on the enzymatic degradation and/or adsorption of insulin onto the subcutaneous tissue at the injection site because SBE4$-\beta$-CyD somewhat inhibited the capillary permeability of FD40.

In conclusion, the present results suggest that SBE4$-\beta$-CyD could be effective in improving insulin bioavailability after subcutaneous administration of insulin solution in rats.

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