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Peak-less hypoglycemic effect of insulin glargine by complexation with maltosyl-β-cyclodextrin

Keiko Uehata[a], Takayuki Anno[a], Kayoko Hayashida[a], Taishi Higashi[a], Keiichi Motoyama[a], Fumitoshi Hirayama[b], Kaneto Uekama[b], Hidetoshi Arima[a,*]

[a] Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
[b] Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

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ABSTRACT

Long-acting insulin products are desired that provide sustained blood glucose lowering without blood glucose level peaks. In the present study, to obtain the more desirable blood glucose lowering effect of long-acting insulin products, we investigated the effect of maltosyl-β-cyclodextrin (G_2-β-CyD) on physicochemical properties and pharmacokinetics/pharmacodynamics of insulin glargine, which is the one of the most widely used insulin analog. G_2-β-CyD increased the solubility and suppressed the aggregation of insulin glargine in phosphate buffer at 9.5, probably due to the interaction of G_2-β-CyD with aromatic residues of the insulin glargine such as tyrosine. In addition, the dissolution rates of insulin glargine from its precipitates were increased by a complexation with G_2-β-CyD. Subcutaneous administration of an insulin glargine solution with G_2-β-CyD to rats gradually decreased blood glucose levels and provided a sustained blood glucose lowering effect without showing the glucose level peaks. These results suggest that G_2-β-CyD can be a useful excipient for sustained release and a truly peak-less formulation of insulin glargine.

1. Introduction

The purpose of treatment of diabetes mellitus is to normalize glycemic control. Normalization of the blood glucose concentration requires control of the plasma insulin profile. Endogenous insulin secretion needs a low basal level of plasma insulin during fasting and an appropriate elevation during meals (Owens and Bolli, 2008). In this context, the intensive insulin therapy is intended to give a basal level and a meal-related bolus level by means of various insulin formulations (Kramer, 1999). Neutral protamine hagedorn insulin (NPH) was mainly used as basal insulin after its launch in 1946 (Owens and Bolli, 2008). However, its duration of action is not long enough to cover the entire day, typically 12 h to 18 h in clinical practice (Heinemann et al., 2000; Lepore et al., 2000). Additionally it shows a peak occurring 4 h to 6 h after subcutaneous injection (Heinemann et al., 2000) and this is connected to an increase of risk of hypoglycemia, particularly nocturnal hypoglycemia following bedtime injection (Fanelli et al., 2002).

In order to overcome these drawbacks, insulin glargine (Lantus®), an insulin analog ($C_{267}H_{404}N_{72}O_{78}S_6$, MW = 6063) was developed by replacing the asparagine at the position of 21 of the A chain with glycine, and two arginines were added to the C-terminus of the B chain in human insulin (Fig. 1). This alteration resulted in low aqueous solubility at neutral pH (Wang et al., 2003). Insulin glargine is supplied in an acidic solution, which becomes neutralized at the injection site, leading to a formation of microprecipitates from which insulin glargine is slowly released into the circulation (Wang et al., 2003). Although insulin glargine provides the blood glucose lowering effect for long time and flatter profile in comparison with NPH, there are still some challenges to minimize the risks of hypoglycemia.

Cyclodextrins (CyDs) are known to form inclusion complexes with various guest molecules (Szente and Szejtli, 1999; Uekama et al., 1998). However, the low aqueous solubility of natural CyDs, especially β-CyD, has restricted their range of applications. To improve their solubility, alkylated, hydroxyl alkylated, sulfobutyl alkylated and branched CyDs have been developed (Stella and Rajewski, 1997; Uekama, 2004; Uekama and Otagiri, 1987). Of these hydrophilic CyDs, maltosyl-β-CyD (G_2-β-CyD), 2-hydroxypropyl-β-CyD (HP-β-CyD) and sulfobutyl ether-β-CyD (SBE-β-CyD) have higher solubility in water and relatively low hemolytic activity, and thus have the potential as pharmaceutical excipients for parenteral preparation (Uekama et al., 1998). In fact, natural β-CyD has a toxic effect on kidney, which is the main organ for removal of CyDs from the systemic circulation and for concentrating CyDs in the proximal convoluted tubule after glomerular filtration (Irie

* Corresponding author. Tel.: +81 96 371 4160; fax: +81 96 371 4420.
E-mail address: arimah@gpo.kumamoto-u.ac.jp (H. Arima).

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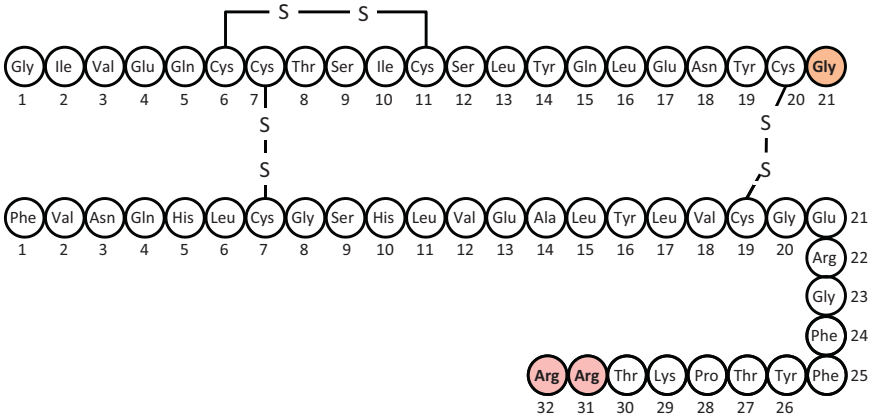

Fig. 1. Secondary chemical structure of insulin glargine.

and Uekama, 1997). On the other hand, highly water-soluble β-CyD derivatives such as G_2-β-CyD, HP-β-CyD and SBE-β-CyD, have very low systemic toxicity, compared with β-CyD.

We previously reported the effects of hydrophilic β-CyDs on the aggregation of bovine insulin in aqueous solution and its adsorption onto hydrophilic surfaces (Tokihiro et al., 1995, 1996, 1997). Of the CyDs tested, G_2-β-CyD potently inhibited insulin aggregation in a neutral solution and its adsorption onto the surfaces of glass and polypropylene tubes. Furthermore, we reported that subcutaneous administration of insulin solution with SBE4-β-CyD to rats maintained higher plasma insulin levels for at least 8 h, possibly due to the inhibitory effects of SBE4-β-CyD on the enzymatic degradation and/or the adsorption of insulin onto the subcutaneous tissue at the injection site (Tokihiro et al., 2000). However, it is still unknown the effects of CyD derivatives on physicochemical properties and pharmacokinetics/pharmacodynamics of insulin analogs including insulin glargine. Recently, we demonstrated that SBE4-β-CyD improved the solubility, dissolution rate and enzymatic resistance of insulin glargine, followed by increase in bioavailability of insulin glargine (Uehata et al., 2011a,b). In the present study, to evaluate the potential use of G_2-β-CyD on not only bioavailability of insulin glargine but also the sustained glucose lowering effect, we examined the effects of G_2-β-CyD on physicochemical properties and pharmacokinetics/pharmacodynamics of insulin glargine in rats.

2. Materials and methods

2.1. Materials

Insulin glargine was a gift from Sanofi-Aventis (Paris, France). G_2-β-CyD was obtained from Ensuiko Sugar Refining (Tokyo, Japan). Recombinant trypsin (EC 3.4.21.4) of proteomics grade was purchased from Roche Diagnostics (Tokyo, Japan). All other materials were of analytical reagent grade, and deionized double-distilled water was used. Phosphate buffer (pH 9.5, $I = 0.2$) was prepared according to the U.S. pharmacopeia: 0.1 mol/L of phosphoric acid solution and 0.1 mol/L of sodium hydroxide solution were mixed, followed by addition of sodium chloride.

2.2. Methods

2.2.1. Spectroscopic studies

Fluorescence and circular dichroism (CD) spectra were measured at 25 °C using a HITACHI fluorescence spectrophotometer F-2500 (Tokyo, Japan) and a JASCO J-720 polarimeter (Tokyo, Japan), respectively. The apparent 1:1 stability constant (K_c) of the insulin glargine/G_2-β-CyD complex was obtained from the Scott's equation (Scott, 1956), assuming the 1:1 guest/host interaction:

$$\frac{[\mathrm{CyD}]_t[\mathrm{G}]_t}{\Delta F} = \frac{1}{K_c\,\varepsilon_c} + \frac{[\mathrm{CyD}]_t}{\varepsilon_c}$$

where $[\mathrm{CyD}]_t$ is the total concentration of G_2-β-CyD, $[\mathrm{G}]_t$ is the total concentration of insulin glargine, ε_c is the difference in fluorescence intensity for free and complexed insulin glargine, and ΔF is the change in fluorescence intensity of insulin glargine by the addition of G_2-β-CyD.

2.2.2. Solubility studies

Excess amounts of insulin glargine were shaken in phosphate buffer (pH 7.4 or 9.5, $I = 0.2$) in the absence and presence of G_2-β-CyD for 5 days at 25 °C. After equilibrium was attained, the solutions were filtered with Millex® GV filter 0.22 μm and dissolved insulin glargine was determined by the high performance liquid chromatography (HPLC) with Agilent 1100 series (Tokyo, Japan) under the following conditions: Merck Superspher® 100 RP-18 column (4 μm, 3 mm × 250 mm, Tokyo, Japan), a mobile phase of phosphate buffer (pH 2.5) and acetonitrile, a gradient flow, increasing the ratio of the acetonitrile (25–40%) over 30 min, a flow rate of 0.55 mL/min, a detection of UV at 214 nm.

2.2.3. Ultrafiltration studies

Ultrafiltration studies were performed using stirred ultrafiltration cells model 8010 (Millipore, Tokyo, Japan) applied with YM30 ultrafiltration discs (MWCO = 30,000) in phosphate buffer (pH 9.5, $I = 0.2$) in the absence and presence of G_2-β-CyD at 25 °C under nitrogen current. Insulin glargine levels in filtrates were determined by HPLC as described above.

2.2.4. Particle size determination

Particle sizes of insulin glargine (0.1 mM) with or without G_2-β-CyD (10 mM) in phosphate buffer (pH 9.5, $I = 0.2$) were measured by Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

2.2.5. Dissolution study of insulin glargine

Insulin glargine (0.1 mM) dissolved in phosphate buffer (pH 9.5, $I = 0.2$) in the absence and presence of G_2-β-CyD (10 mM) was precipitated by a pH shift to 7.4. After centrifugation (2500 rpm, 10 min) the supernatant was discarded, and then phosphate buffer (pH 7.4, $I = 0.2$) was newly added to the precipitate at 25 °C. At appropriate intervals, an aliquot of the dissolution medium was withdrawn, centrifuged at 2500 rpm for 10 min, and analyzed for the insulin glargine by HPLC as described above.

2.2.6. Stability of insulin glargine against tryptic cleavage

Insulin glargine (0.1 mM) in phosphate buffer (pH 9.5, $I = 0.2$) was incubated with recombinant trypsin (0.02 mg/mL) in the absence and presence of G_2-β-CyD at 37 °C. At appropriate intervals, 5 μL of sample solution was withdrawn and determined intact insulin glargine level by HPLC. The rate constant (k_c) and stability constant (K_c) of an apparent 1:1 complex of insulin glargine with G_2-β-CyD under the tryptic cleavage were determined by quantitative analysis according to the following equation (Ikeda et al., 1975):

$$\frac{[CyD]_t}{k_0 - k_{obs}} = \frac{1}{k_0 - k_c} \cdot [CyD]_t + \frac{1}{K_c \cdot (k_0 - k_c)}$$

where k_0, k_{obs} and $[CyD]_t$ stand for the rate constant without CyD, the apparent rate constant and the total concentration of CyD, respectively.

2.2.7. Subcutaneous administration of insulin glargine/G_2-β-CyD complex to rats

Serum insulin glargine and glucose levels of rats were measured by the enzyme immunoassay and the mutarotase-glucose oxidase method, respectively. The solution (0.582 mL/kg) of insulin glargine (2 IU/kg) in phosphate buffer (pH 9.5, $I = 0.2$) in the absence and presence of G_2-β-CyD (100 mM) was subcutaneously injected in male Wistar rats (200–250 g), and at appropriate intervals blood samples were taken from the jugular veins. Serum insulin glargine and glucose were determined by Glyzyme Insulin-EIA Test Wako (Wako Pure Chemicals Ind., Osaka, Japan) and Glucose-CII-Test Wako (Wako Pure Chemicals Ind., Osaka, Japan), respectively. Serum glucose levels after the administration of insulin glargine/G_2-β-CyD solution were expressed as a percentage of the initial glucose level before injection. All animal experiments were approved by the Animal Study Committee of the Kumamoto University, Faculty of Life Sciences (Approval number: H21-233).

2.2.8. Statistical analysis

Data are given as the mean ± S.E.M. Statistical significance of means for the studies was determined by analysis of variance followed by Scheffe's test. p-Values for significance were set at 0.05.

3. Results and discussion

3.1. Spectroscopic studies

We previously reported that G_2-β-CyD inhibited the insulin aggregation in neutral solution, possibly due to the inclusion of G_2-β-CyD with aromatic side chains of insulin, such as B26-tyrosine, A19-tyrosine, B1-phenylalanine and B25-phenylalanine (Tokihiro et al., 1996). In the present study, to reveal whether G_2-β-CyD interacts with insulin glargine, we investigated the effects of G_2-β-CyD (10 or 100 mM) on the fluorescence and CD spectrum of insulin glargine (0.1 mM) (Fig. 2). To obtain the clear solution of insulin glargine (0.1 mM) in spectroscopic studies, insulin glargine with G_2-β-CyD was dissolved in phosphate buffer (pH 9.5, $I = 0.2$) at 25 °C. In our preliminary study, we confirmed that insulin glargine dissolved in phosphate buffer (pH 9.5, $I = 0.2$) was relatively chemically stable, remaining 91.0% of insulin glargine after 6 days storage at 25 °C, in comparison with the initial concentration (data not shown). The fluorescence intensity of tyrosine of insulin glargine at 306 nm was slightly enhanced by the addition of G_2-β-CyD (10 mM) (Fig. 2A).

Next, the titration curve of the fluorescence intensity of insulin glargine against a concentration of G_2-β-CyD was taken, and the Scott's plot showed a straight line, suggesting that G_2-β-CyD forms the 1:1 complex with insulin glargine. Herein, G_2-β-CyD was previously reported to form the 1:1 complex with insulin in solution,

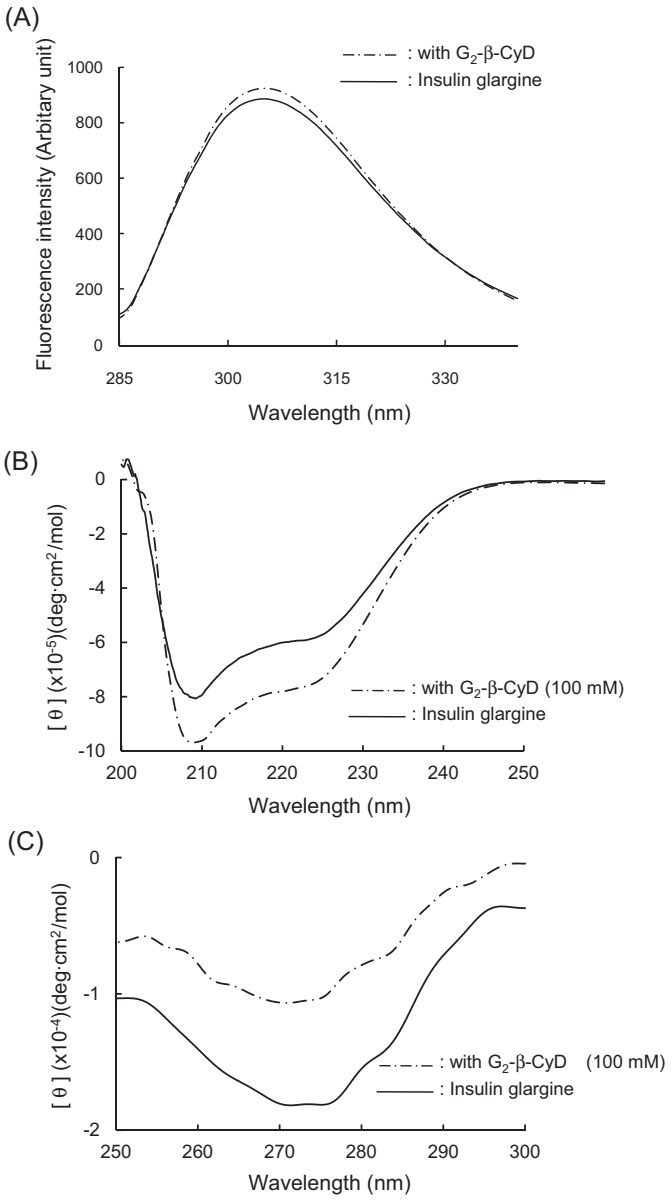

Fig. 2. Effect of G_2-β-CyD (10 mM (A) and 100 mM (B and C)) on fluorescence spectrum (A) and CD spectrum (B and C) of insulin glargine (0.1 mM) in phosphate buffer (pH 9.5, $I = 0.2$) at 25 °C. The excitation wavelength in measurement of fluorescence spectrum was 277 nm.

and the complexation should be initiated by the inclusion of one of the various binding sites on the insulin molecule into G_2-β-CyD, which may in turn prevent the further access of the second G_2-β-CyD to the other binding sites, probably due to steric hindrance and/or conformational changes of the polypeptide (Tokihiro et al., 1997). Collectively, we assumed that G_2-β-CyD forms the 1:1 complex with insulin glargine. The apparent 1:1 stability constant (K_c) of the insulin glargine/G_2-β-CyD complex, determined by the titration curve of the fluorescence intensity against a concentration of G_2-β-CyD with the Scott's equation (Scott, 1956) was calculated to be $27 \pm 2\ M^{-1}$, suggesting a weak interaction of G_2-β-CyD with the aromatic amino acid residues of insulin glargine.

In the CD spectroscopic studies, two negative bands at 208 and 225 nm were significantly increased, while the negative band at 273 nm was decreased by addition of G_2-β-CyD (Fig. 2B and C). The negative bands at 208 and 225 nm are assigned to α-helical (a characteristic feature of the monomer) and β-structure (a predominant

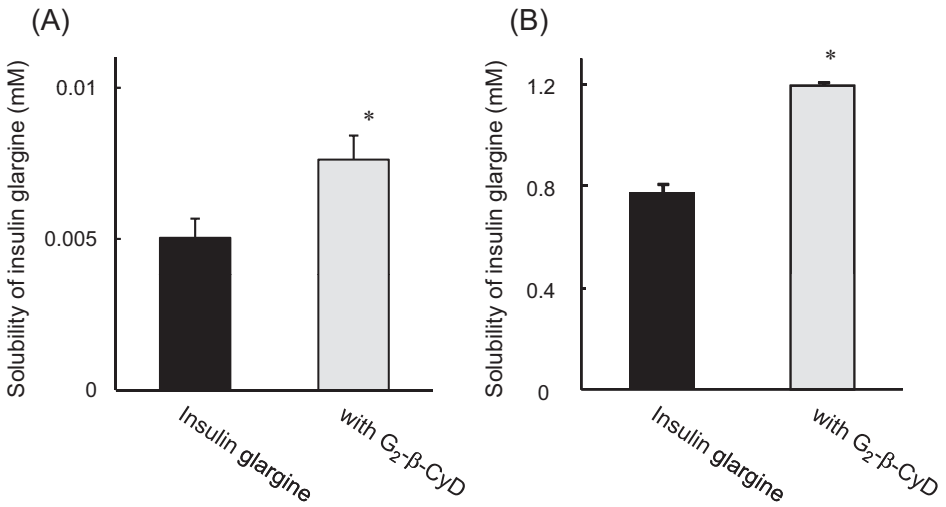

Fig. 3. Effect of G_2-β-CyD (10 mM) on solubility of insulin glargine in phosphate buffer ((A) pH 7.5 and (B) pH 9.5, $I = 0.2$) at 25 °C. The concentration of insulin glargine was determined by HPLC. Each value represents the mean ± S.E.M. of 3 experiments. $^*p < 0.05$, compared to insulin glargine.

feature of dimer), respectively (Goldman and Carpenter, 1974). Furthermore, negative band at 273 nm is assigned to aromatic amino residues (tyrosine and phenylalanine) which exhibit optical activity as a function of aggregation of insulin molecule (Goldman and Carpenter, 1974). These alterations of CD spectra suggest that G_2-β-CyD increased a monomer and a dimer of insulin glargine and decreased its aggregation.

3.2. Solubility studies

Currently subcutaneous injection of solution, not suspension, is the usual route for administration of insulin and its analogs. Therefore, the effect of G_2-β-CyD on the solubility of insulin glargine was examined at pH 7.4 and 9.5. As shown in Fig. 3, the solubility of insulin glargine in phosphate buffer at pH 7.4 and 9.5 was significantly increased by the addition of G_2-β-CyD. It is estimated that an increase in solubility of insulin glargine was caused by the complexation with G_2-β-CyD. These results suggest that G_2-β-CyD potentially enhances solubility of insulin glargine in phosphate buffer at pH 7.4 and 9.5.

3.3. Ultrafiltration studies

To estimate the effects of G_2-β-CyD on aggregation of insulin glargine, we performed ultrafiltration studies using the membrane YM30 (MWCO = 30,000) in phosphate buffer (pH 9.5, $I = 0.2$). As shown in Fig. 4, insulin glargine permeated through the ultrafiltration membrane by 48%. On the other hand, G_2-β-CyD slightly enhanced the permeation of insulin glargine up to 55% with a significant difference, compared to insulin glargine alone. These results suggest that G_2-β-CyD leads to dissociation of soluble multimers of insulin glargine, such as hexamer and/or higher order oligomer (MW > 30,000). Following the ultrafiltration experiment, particle sizes of insulin glargine were determined in the absence and presence of G_2-β-CyD (Table 1). There were no significant difference in particle sizes of insulin glargine in the absence and presence of G_2-β-CyD in phosphate buffer (pH 9.5, $I = 0.2$). These results suggest the potential use of G_2-β-CyD as an aggregation-inhibitor for insulin glargine without remarkable influence on the particle size of insulin glargine.

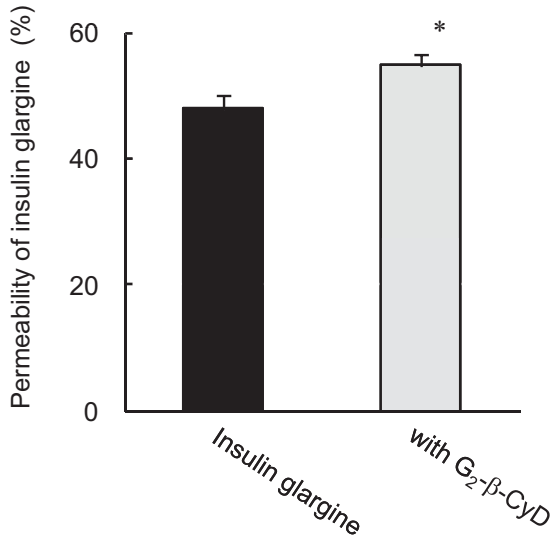

Fig. 4. Effect of G_2-β-CyD (10 mM) on permeation of insulin glargine (0.1 mM) through the ultrafiltration membrane having nominal molecular weight limit of 30,000 in phosphate buffer (pH 9.5, $I = 0.2$) at 25 °C. The concentration of insulin glargine was determined by HPLC. Each value represents the mean ± S.E.M. of 5–17 experiments. $^*p < 0.05$, compared to insulin glargine.

3.4. Dissolution study of insulin glargine

In order to investigate the effects of G_2-β-CyD on the sustained release of insulin glargine, the dissolution rate of insulin glargine from isoelectric precipitates formed in the absence and presence of G_2-β-CyD was determined (Fig. 5). G_2-β-CyD significantly increased the dissolution rate of insulin glargine after 24 h, compare to insulin glargine alone. This enhancing effect of

Table 1
Particle size of insulin glargine with or without G_2-β-CyD (10 mM) in phosphate buffer (pH 9.5).

System	Diameter (nm)
Insulin glargine	744 ± 82
with G_2-β-CyD	796 ± 82

The particle size was measured by Zetasizer Nano. The concentrations of insulin glargine and G_2-β-CyD were 0.1 mM and 10 mM, respectively.
Each value represents the mean ± S.E.M. of 6–9 experiments.

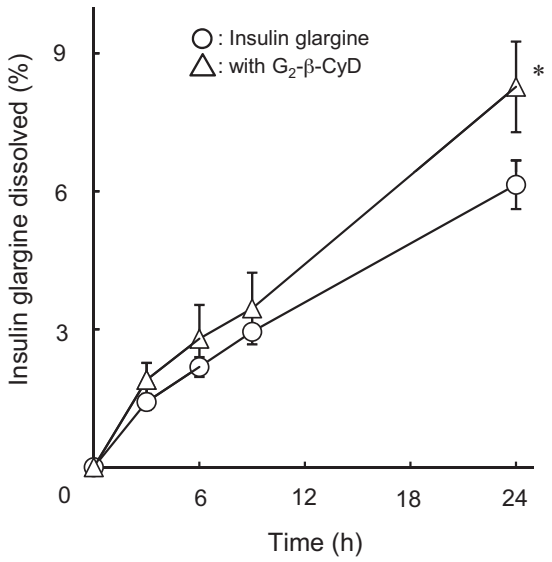

Fig. 5. Effect of G_2-β-CyD (10 mM) on the dissolution rate from isoelectric precipitation of insulin glargine in phosphate buffer (pH 9.5, $I = 0.2$) at 25 °C. The initial concentration of insulin glargine was 0.1 mM, and then precipitated at pH 7.4. The concentration of insulin glargine was determined by HPLC. Each point represents the mean ± S.E.M. of 3 experiments. $^*p < 0.05$, compared to insulin glargine.

G_2-β-CyD is clearly consistent with its solubilizing effect as shown in Fig. 3. These results suggest that G_2-β-CyD increases the dissolution property of insulin glargine.

3.5. Stability of insulin glargine against tryptic cleavage

Insulin and its analogs are digested by proteases such as trypsin, which cleaves insulin at the carboxyl side of residues B29-lysine and B22-arginine, at injection site and in systemic circulation (Schilling and Mitra, 1991). Therefore, a resistance toward enzymatic degradation is required for insulin or its analogs formulation to improve their bioavailability. Next, we investigated the effects of G_2-β-CyD on enzymatic stability of insulin glargine against trypsin digestion. As shown in Fig. 6A, the apparent degradation rate constant of insulin glargine in the absence of G_2-β-CyD (k_0) was $0.357 \pm 0.004\ h^{-1}$. Furthermore, the apparent rate constant (k_{obs}) in the presence of G_2-β-CyD decreased as the G_2-β-CyD concentration increased. The degradation rate constant (k_c) and stability constant (K_c) of the apparent 1:1 complex calculated from the regression lines shown in the Fig. 6B were $0.207 \pm 0.023\ h^{-1}$ and $563 \pm 139\ M^{-1}$, respectively. The aromatic amino acid residues in the insulin glargine molecule, which are capable of interacting with G_2-β-CyD (e.g. B25-phenylalanine or B26-tyrosine), locate near the three tryptic digestive sites in insulin glargine (B22–B23, B29–B30 and B31–B32). The K_c of apparent 1:1 complex of insulin glargine and G_2-β-CyD determined by the tryptic cleavage study did not agree with that by the fluorescence study. The reason for the difference in K_c value is thought to be due to the difference in the experimental conditions such as temperature of the solutions (25 °C for the spectroscopic study and 37 °C for the tryptic cleavage study) and the presence of trypsin. These results indicate that the stabilization of insulin glargine to tryptic cleavage was caused by a complexation with G_2-β-CyD. Furthermore, the interaction between G_2-β-CyD and trypsin may be attributed to the inhibition of enzymatic degradation of insulin glargine by trypsin.

3.6. Subcutaneous administration of insulin glargine/G_2-β-CyD solution to rats

We evaluated the effects of G_2-β-CyD on pharmacokinetics and pharmacodynamics of insulin glargine after subcutaneous injection to rats. Fig. 7A and Table 2 show the serum insulin glargine level-time profiles and pharmacokinetic parameters, respectively, after subcutaneous administration of insulin glargine (2 IU/kg) with or without G_2-β-CyD (100 mM) in phosphate buffer (pH 9.5) to rats. When insulin glargine was injected, the time (T_{max}) required to reach maximum level (C_{max}) of insulin glargine was at 1.20 h after injection, and then the serum insulin glargine level decreased to the basal level. On the other hand, T_{max} in the G_2-β-CyD system significantly delayed to 5.82 h, although C_{max} was the same as that of insulin glargine alone. The area under the serum insulin glargine level-time curve (AUC) up to 12 h in the G_2-β-CyD system (AUC = 732.25 μU/mL h) was significantly increased, compared to those of insulin glargine alone (AUC = 596.80 μU/mL h).

Next, to investigate the influence of G_2-β-CyD on the hypoglycemic effect of insulin glargine, we evaluated the serum glucose level-time profiles (Fig. 7B) and pharmacodynamics parameters (Table 3) after subcutaneous administration of insulin glargine (2 IU/kg) with or without G_2-β-CyD (100 mM) in phosphate buffer (pH 9.5) to rats. Herein, serum glucose levels were not statistically significant difference at time 0 between the insulin alone system and the G_2-β-CyD system, indicating that the both groups were

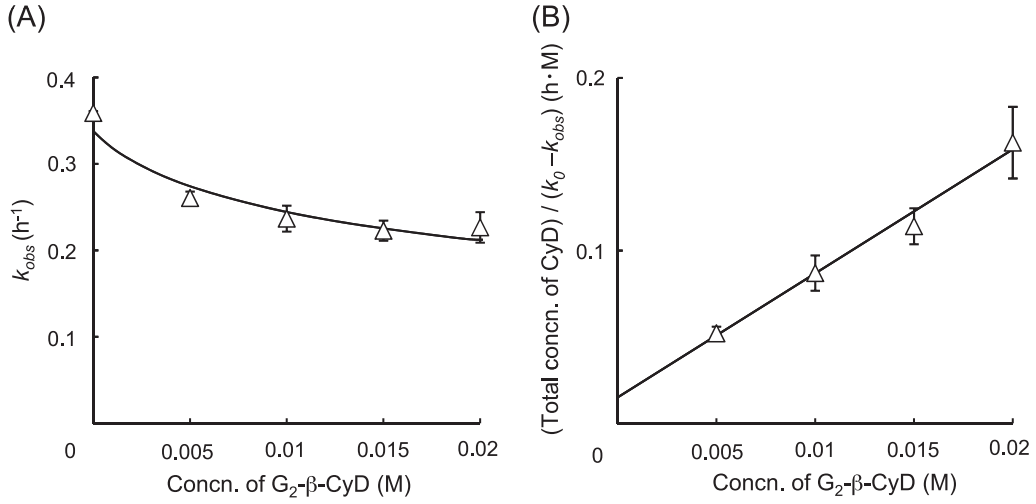

Fig. 6. Effects of G_2-β-CyD (5–20 mM) on tryptic cleavage (2 IU) of insulin glargine (0.1 mM) in phosphate buffer (pH 9.5, $I = 0.2$) at 37 °C. The concentration of insulin glargine was determined by HPLC. Each point represents the mean ± S.E.M. of 3 experiments.

Table 2
In vivo pharmacokinetics parameters of insulin glargine with or without G_2-β-CyD (100 mM).

System	T_{max}[a] (h)	C_{max}[b] (μU/mL)	AUC[c] (μU/mL h)
Insulin glargine	1.20 ± 0.13	124.30 ± 12.81	596.80 ± 36.55
Insulin glargine/G_2-β-CyD	5.82 ± 0.18*	130.36 ± 11.68	732.25 ± 33.25*

Each value represents the mean ± S.E.M. of 6–9 experiments.
[a] Time required to reach the maximum serum insulin glargine level.
[b] Maximum serum insulin glargine level.
[c] Area under the serum insulin glargine level-time curve up to 12 h post-administration.
* $p < 0.05$, compared to insulin glargine.

Table 3
In vivo pharmacodynamics parameters of insulin glargine with or without G_2-β-CyD (100 mM).

System	T_{nadir}[a] (h)	C_{nadir}[b] (%)	AUC_G[c] (%h)
Insulin glargine	1.55 ± 0.16	48.31 ± 43.75	389.21 ± 22.46
Insulin glargine /G_2-β-CyD	5.60 ± 1.05*	67.60 ± 3.86*	341.89 ± 32.92

Each value represents the mean ± S.E.M. of 7–11 experiments.
[a] Time to nadir blood glucose concentration.
[b] Nadir blood glucose concentration.
[c] The cumulative percentage of change in serum glucose levels up to 12 h post-administration.
* $p < 0.05$, compared to insulin glargine.

under the same experimental conditions in terms of a decrease in the serum glucose level. Additionally, the glucose levels were not changed by the subcutaneous administration of the buffer in the presence and absence of G_2-β-CyD (100 mM) alone (data not shown). When insulin glargine alone was injected, the time (T_{nadir}) required to reach minimum glucose level (C_{nadir}) was 1.55 h after injection, and the serum glucose levels recovered within 6 h to ca. 80% of the control levels. On the other hand, T_{nadir} increased significantly in the G_2-β-CyD system, while the area under serum glucose level-time curve (AUC_G) did not change notably, compared to insulin glargine alone. Importantly, the addition of G_2-β-CyD provided the sustained effect to insulin glargine without clear peaks of blood-glucose level. Such a peak-less profile of the blood glucose level would decrease risks of hypoglycemia, which provides patients with a better glycemic control and a higher quality of life. In addition, the low glucose level around ca. 80% of the control levels in the G_2-β-CyD system is expected to continue over a 12 h period, although the required serum glucose level and the end point were not principally determined under the present experimental conditions.

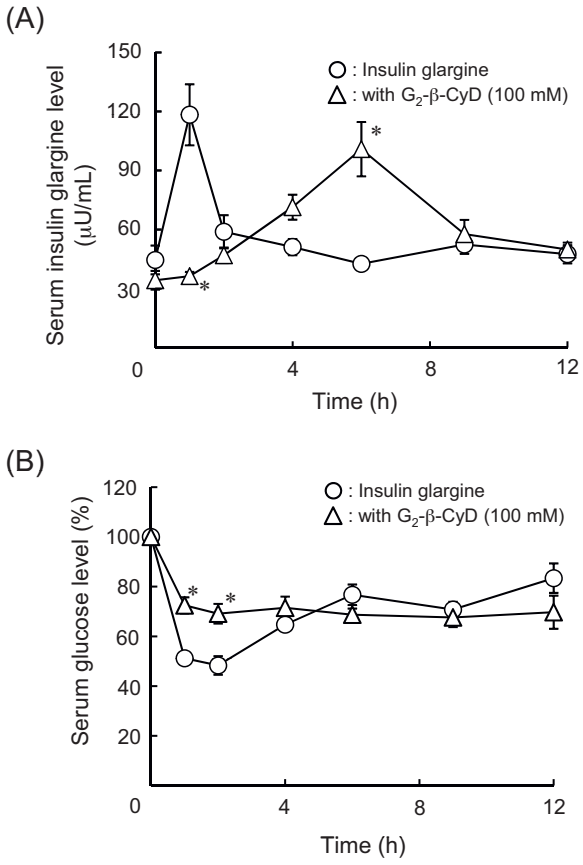

Fig. 7. Effects of G_2-β-CyD (100 mM) on serum insulin glargine (A) and glucose (B) levels after subcutaneous administration of insulin glargine (2 IU/kg) to rats. Each point represents the mean ± S.E.M. of 6–11 experiments. *$p < 0.05$, compared to insulin glargine.

This sustained effect of G_2-β-CyD to insulin glargine without clear peaks of blood-glucose level was unique, because this effect was not observed in the system of insulin glargine with β-CyD, SBE4-β-CyD (Uehata et al., 2011a,b), SBE7-β-CyD (Uehata et al., 2011b) or sulfate-β-CyD (Uehata et al., 2011b). Hence, the branched maltosyl moiety as well as the hydrophobic cavity in the G_2-β-CyD molecule may be involved in the preferable sustained effect. However, the reason for this peak-less profile of the blood glucose level is still unclear. This effect may be explained by the following three mechanisms. Firstly, this peak-less profile of the blood glucose level was not observed in the insulin/G_2-β-CyD system (Tokihiro et al., 2000), suggesting that the profile is specific to insulin glargine, not insulin. Hence, it is possible that G_2-β-CyD may somewhat affect differential properties of insulin glargine such as isoelectric precipitation and/or adsorption of insulin glargine on subcutaneous tissue in the injection site. Secondly, sodium glycocholate, a metabolic product of cholesterol, is known to dissociate insulin oligomers to monomer (Uchiyama et al., 1993). In addition, it was reported that insulin interacts with phospholipids (Perry et al., 1971). These lines of evidence make it tempting to speculate that G_2-β-CyD may strongly inhibit the interaction between insulin glargine and intravital compounds such as cholesterol and/or phospholipids, resulting in acceleration of the formation of insulin glargine oligomers *in vivo* (Fig. 2B and C). Thirdly, the stability constant of insulin glargine with G_2-β-CyD ($563 \pm 139\ M^{-1}$) calculated from tryptic cleavage was higher than those of SBE4-β-CyD ($144 \pm 18\ M^{-1}$), SBE7-β-CyD ($182 \pm 22\ M^{-1}$) and sulfate-β-CyD ($244 \pm 44\ M^{-1}$). Thereby, this potent stabilizing effect of G_2-β-CyD on enzymatic degradation could be ascribed to the long retention of insulin glargine in subcutaneous tissue. Collectively, the complicated relationship between these factors might be associated with the preferable peak-less

hypoglycemic effect of insulin glargine by complexation with G_2-β-CyD. Thereafter, to gain insight into the mechanism for the desirable pharmacodynamic effect of the insulin glargine/G_2-β-CyD system, further elaborate studies are required.

In conclusion, in the present study, we revealed that G_2-β-CyD provided adequate physicochemical properties and a peak-less sustained hypoglycemic effect of insulin glargine after subcutaneous injection to rats. These findings indicate that G_2-β-CyD could be a useful excipient providing desirable sustained release profile to insulin glargine.

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