Selected Paper

Antidiabetic Activity of Zn(II) Complexes with a Derivative of L-Glutamine

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The insulinomimetic activity of zinc(II) complexes Zn(gln-m)$_2$ and Zn(gln-e)$_2$, respectively, with L-γ-glutamylmethylamide (gln-m) and -ethylamide (theanine: gln-e), components of green tea, were compared to that of Zn(gln)$_2$ with L-glutamine (gln) in an in vitro study using isolated rodent adipocytes treated with epinephrine in the presence of glucose, expressed as the IC_{50} value (50% inhibition concentration) of free fatty acids (FFA) released from fat cells. The former were found to have higher inslinomimetic activity than that of the latter. The IC_{50} values of the former were 0.61 and 0.56, respectively. Then, the anti-diabetic activity of a zinc(II) complex, Zn(gln-e)$_2$, was examined in an in vivo experiment on KK-A^y mice, model animals of type-2 DM, by the intraperitoneal (ip) injection of 3–4 mg Zn/kg b.w./day for 13 days. The blood-glucose level of a group treated with a Zn complex, Zn(gln-e)$_2$, decreased significantly after 13 days compared to those of non-treated and gln-e treated groups. The serum concentrations of triglyceride (TG) and HbA_{1c} decreased significantly in Zn(gln-e)$_2$ treated KK-A^y mice compared to those of the gln-e treated group, respectively. Furthermore, the improvement in glucose tolerance was confirmed by an oral glucose tolerance test.

An increasing number of people suffering from diabetes mellitus (DM: type 1 and type 2, but mainly type 2) is a worldwide problem. The latest (2000) WHO estimate for the number of people with diabetes worldwide is 177 million, and is predicted to increase to at least 300 million by 2025.[1]

In Japan,[2] the number of people with a "high possibility of developing diabetes" (HbA_{1c} higher than 6.1%) has increased from 6,900,000 to 7,400,000 during these five years, the prevalence being about 9.0% according to a 2002 report of the Ministry of Health, Labor, and Welfare, Japan. Those with a "possible or pre-diabetic" tendency (HbA_{1c} 5.6%–6.1%) has also increased to about 30% compared to the figures in 1997.

Thus, several types of medicines for treating non-insulin-dependent type 2 DM have been developed worldwide. However, such therapeutic medicines have been reported to have some problems, including side effects. As a consequence, it is desired to develop clinically useful drugs with low toxicity.

The Zn^{2+} ion is known to be one of the most important essential trace elements found in living organisms as well as in many metalloproteins and metalloenzymes.[3] Among the many pharmacological and nutritional roles of the Zn^{2+} ion,[4] this metal ion was found in 1980 to stimulate lipogenesis in the adipocytes of rats, which was considered to be similar to the action of insulin.[5] Following this finding, several researchers attempted to confirm the insulinomimetic activity of the Zn^{2+} ion.[6–8] For example, Shisheva et al. reported that the Zn^{2+} ion stimulated glucose uptake and lipid synthesis in the adipocytes of rats, and that the oral administration of a high dose of $ZnCl_2$ (210 mg/kg body weight) to insulin-dependent type 1 model diabetic rats treated by streptozotocin reduced blood-glucose levels by as much as 50%.[7] On the other hand, Chen et al. found the hypoglycemic effect of $ZnCl_2$ (20 mM) in type 2 diabetic *ob*/*ob* mice.[8] However, Zn(II) complexes have never been examined.

It has been reported that the bis(maltolato)Zn(II) complex, Zn(mal)$_2$, increases zinc absorption into erythrocytes more than a dose of free Zn^{2+} ion.[9] Recently, we found that Zn(mal)$_2$ with a Zn(O$_4$) coordination mode has a higher insulinomimetic activity than free Zn^{2+} ions, as estimated in in vitro experiments.[10] In addition, we have reported that Zn(II) complexes with Zn(O$_4$), Zn(N$_2$O$_2$), Zn(N$_2$S$_2$), Zn(O$_2$S$_2$), and Zn(S$_4$) coordination modes have high insulinomimetic activities with regard to in vitro experiments and blood glucose lowering effects.[11–19] L-Glutamine (gln), an amino acid, is a component of glutathione, the body's primary antioxidant, which is present in virtually every cell. If you are deficient in gln, you are likely to be deficient in glutathione. What is fascinating about gln is that a handful of substances can naturally boost the level of human growth hormone secretion, essential for normal growth and development. Recently, growth hormones have been used experimentally for treating muscle loss, which often occurs among invalids and elderly prone to the wasting syndrome.[20]

Theanine (L-γ-glutamylethylamide = gln-e) and L-γ-glu-

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tamylmethylamide (gln-m) are alkylated derivatives of an amide proton of gln. The gln-e and gln-m are known to be one of the main components of Japanese green tea, and to exhibit a hypotensive effect in spontaneously hypertensive rats.[21] Also, gln-e is reported to depress a rat's liver disorder.[22]

In this study, we synthesized Zn(II) complexes, $Zn(gln\text{-}e)_2$ and $Zn(gln\text{-}m)_2$ with gln-e and gln-m, respectively, characterized by NMR spectral, physical, and analytical data, and estimated the insulinomimetic activities in vitro in relation to the inhibition of FFA release from isolated rat adipocytes treated with epinephrine by comparisons with Zn(II) complexes, $Zn(asn)_2$ and $Zn(gln)_2$, with L-asparagine (asn) and gln, respectively. A moderate enhancement of the lipophilicity of ligands can be expected to enhance insulinomimetic activity.[13,23,24]

We also observed antidiabetic activity by in vivo experiments, such as by ip injection of $Zn(gln\text{-}e)_2$ on the animal model of type-2 DM, KK-A^y mice, and compared it to free gln-e treated and non-treated control mice.

Experimental

Materials. All purchased reagents were of analytical or reagent grade, and used without purification. Elemental analyses of the complexes were carried out on a Perkin-Elmer 240C elemental analyzer (Tokyo, Japan). FT-IR spectra were recorded on a JASCO FT/IR-420 spectrophotometer (Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were recorded on JEOL GX-400 FT-NMR spectrometers (Tokyo, Japan) and DSS was used as an internal standard in a D_2O solution. A small amount of NaOD or DCl in the D_2O solution was used for pH adjustment. The melting points were determined on a Yanaco MP-J3 apparatus (Kyoto, Japan). Specific rotations $[\alpha]_D$ were measured on a Jasco DIP 300 polarimeter (Tokyo, Japan).

Preparation of Ligand. A ligand, gln-m, was prepared by a similar method to a reference.[25] A Cu(II) complex with pyroglutamate was prepared by pyroglutamic acid and basic cupper carbonate. The Cu(II) complex was treated with a 40% methylamine/methanol solution at 70 °C for 7 d in an autoclave. The solvent was removed under reduced pressure, after which the residue was dissolved in water, then treated with H_2S gas; the deposited copper sulfide was then filtrated out. The filtrate was evaporated under reduced pressure and washed with methanol.

The compound was obtained as a white powder. Yield: 26%. mp: 183–186 °C. IR (KBr): 1649 and 1581 cm^{-1} for $\nu_{C=O}$ (amide and carboxylate, respectively). ^{1}H NMR (D_2O, pD = 8.3): δ 2.11 (dd, 2H, J = 14.9, 7.4 Hz), 2.34–2.46 (m, 2H), 2.73 (s, 3H), and 3.75 (t, 1H, J = 6.2 Hz). Anal. Calcd for $C_6H_{12}N_2O_3 \cdot 0.2\,H_2O$: C, 44.00; H, 7.63; N, 17.10%. Found: C, 44.12; H, 7.45; N, 16.83%. $[\alpha]_D$: +7.1° (H_2O). FAB MS: m/z 161 $[M + H]^+$.

Preparation of Complexes. The Zn(II) complexes used in this study were readily prepared by adding $ZnCl_2$ to a methanol solution of a lithium salt of an appropriate ligand (generated in situ from ligands and lithium hydroxide) at room temperature. All complexes were purified, washed with methanol, identified by elemental analyses and IR and ^{1}H NMR spectra, and found to be molecular complexes without counter ions. $Zn(gln\text{-}m)_2$: Yield: 63%. mp: 255–284 °C (dec.). IR (KBr): 1641 and 1604 cm^{-1} for $\nu_{C=O}$ (amide and carboxylate, respectively). ^{1}H NMR (D_2O, pD = 8.3): δ 1.98 (m, 1H), 2.16 (m, 1H), 2.40 (t, 2H, J = 7.7 Hz), 2.74 (s, 3H), and 3.51 (s, 1H). Anal. Calcd for $Zn(C_6H_{11}N_2O_3)_2 \cdot 0.8H_2O$: C, 36.20; H, 5.97; N, 14.07%. Found: C, 36.25; H, 5.57; N, 13.76%. $[\alpha]_D$: −5.4° (H_2O). $Zn(gln\text{-}e)_2$: Yield: 93%. mp: 281–289 °C (dec.). IR (KBr): 1637 and 1606 cm^{-1} for $\nu_{C=O}$ (amide and carboxylate, respectively). ^{1}H NMR (D_2O, pD = 8.5): δ 1.11 (t, 3H, J = 7.3 Hz), 1.97 (m, 1H), 2.15 (m, 1H), 2.39 (t, 2H, J = 7.8 Hz), 3.20 (q, 2H, J = 7.3 Hz), and 3.50 (dd, 1H, J = 7.3, 5.1 Hz). Anal. Calcd for $Zn(C_7H_{13}N_2O_3)_2$: C, 40.84; H, 6.36; N, 13.61%. Found: C, 40.69; H, 6.33; N, 13.37%. $[\alpha]_D$: −11.3° (H_2O).

Measurement of Inhibitory Activity of Zn(II) Complexes in vitro on the Release of Free Fatty Acid (FFA) Release from Isolated Rodent Adipocytes by Epinephrine. Isolated adipocytes of male Wistar rat (1.0×10^6 cells/mL), prepared as described,[26] were preincubated at 37 °C for 30 min at various concentrations (10^{-4}–10^{-3} M) of each Zn(II) complex in KRB buffer (120 mM NaCl, 1.27 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.75 mM KCl, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, and 5 mM glucose: pH 7.4) containing 2% BSA. A 10^{-4} M epinephrine was then added to the reaction mixtures, and the resulting solutions were incubated at 37 °C for 180 min. The reactions were stopped by soaking in ice water, and the mixtures were centrifuged at 3000 rpm for 10 min. The FFA levels for the outer solution of the cells were determined with an FFA kit (Wako Pure Chemical Industries). The IC_{50} values were obtained based on the concentration of the Zn(II) complex to inhibit 50% of the FFA released from the adipocytes.

Experimental Animals for in vivo Experiment. Male KK-A^y mice aged 4 weeks (body weight 16–19 g) with type 2 diabetes were obtained from CLEA Japan Inc. These mice were housed individually in stainless steel cages in an air conditioned room (23 ± 2 °C) with a 12-h light and dark cycle (lighting from 7:00 to 19:00) and given free access to solid food (CE-2: CLEA Japan Inc.) and tap water. All in vivo experiments were approved by the Experimental Animal Research Committee at Kobe Women's University.

These mice were divided into three groups of 5 mice each after the onset of type 2 diabetes (9 weeks of age): an untreated group, a gln-e treated group and a $Zn(gln\text{-}e)_2$ treated group. They were given daily ip injections of each material at about 10:00 a.m. after measuring their blood glucose levels, for a period of about 2 weeks from 9 to 11 weeks of age. KK-A^y mice were given daily ip injections of $Zn(gln\text{-}e)_2$ at a dose of 3 mg Zn/kg of body weight[27] for the first 7 days. The gln-e group was dosed with the same amount as that of the gln-e as the $Zn(gln\text{-}e)_2$ treated group, and the control group was given the same amount of water as the $Zn(gln\text{-}e)_2$ solution.

The dose of $Zn(gln\text{-}e)_2$ was changed, depending on the daily changes in the blood glucose levels of the mice from the 8th day. For example, when the blood-glucose level was <350 or >350, the dose was changed to 3 and 4 mg Zn/kg of the body weight, respectively. The blood sample for the analysis of the glucose levels was obtained from the tail vein of each mouse, and measured using a portable blood-glucose meter (Glucocard: Arkray, Kyoto, Japan). The body weights of the KK-A^y mice and the intakes of solid food and drinking water for each mouse were measured daily throughout the experiments.

Hematological Analysis. Blood samples for analyses of the levels of triglyceride (TG), blood urea nitrogen (BUN), alanine aminotranferease (ALT), aspartate aminotransferase (AST), and total cholesterol (TCHO) were obtained from orbital exsanguination under ether anesthesia.

The serum concentrations of TG, BUN, ALT, AST, and TCHO were determined from measurements courted by Fuji Dry Chem (Fuji Medical Co., Tokyo, Japan). The Hemoglobin A_{1c} level

Table 1. ^{13}C NMR Data of Each Ligand and Complexes, $Zn(gln)_2$, $Zn(gln\text{-}m)_2$, $Zn(gln\text{-}e)_2$, and $Zn(asn)_2$ in D_2O at 27 °C

Compound	pD	Chemical shifts (ppm), $\delta(\Delta\delta^{a)})$						
		$C^{carbonyl}$	C^{α}	C^{β}	C^{γ}	C^{amide}	C^{alkyl}	
Gln	8.7	177.6	57.1	29.5	33.8	180.6		
$Zn(gln)_2$	8.7	182.1	56.5	31.4	34.5	181.2		
		(4.5)	(−0.6)	(1.9)	(0.7)	(0.6)		
Gln-m	8.3	177.4	57.1	29.6	34.4	178.1	28.8	
$Zn(gln\text{-}m)_2$	8.3	182.0	56.5	31.6	35.1	178.6	28.8	
		(4.6)	(−0.6)	(2.0)	(0.7)	(0.5)	(0.0)	
Gln-e	8.5	177.5	57.2	29.8	34.6	177.3	37.4	16.2
$Zn(gln\text{-}e)_2$	8.5	182.3	56.4	31.7	35.3	177.8	37.4	16.3
		(4.8)	(−0.8)	(1.9)	(0.7)	(0.5)	(0.0)	(0.1)
asn	8.2	177.0	54.3	38.0	—	177.5		
$Zn(asn)_2$	8.2	181.7	53.8	39.9	—	178.6		
		(4.7)	(−0.5)	(1.9)		(1.1)		

a) $\Delta\delta = \delta$ [Zn(II) complex] − (free ligand).

was measured by an immunoassay method (DCA 2000 System, Bayer Co., Ltd., Tokyo, Japan) before and after administration of $Zn(glu\text{-}e)_2$.

Oral Glucose Tolerance Test (OGTT). After administration of $Zn(gln\text{-}e)_2$ for 13 days, the mice were fasted for 16 h and then glucose at a dose of 1 g/kg body weight was given orally. Blood samples for analysis of glucose levels were obtained from the caudal vein at 0, 30, 60, 90, 120, and 180 min after glucose administration, and blood glucose concentrations were measured with the Glucocard.

Statistical Analysis. Data are expressed as the means ± standard deviations (S.D.). Statistical analysis was performed using the Student's *t*-test at 5% or 1% significant level of the difference.

Results and Discussion

The Structures of Zn(II) Complexes. The molecular structure of the $[Zn(asn)_2]_n$ complex, was reported to by Stephens et al. have a distorted octahedral environment.[28] The carboxylic O atom and the α-amino N atom from each ligand coordinate to the Zn atom in a trans square–planar coordination and octahedral environment is completed by carbonyl O atoms from neighbouring molecules, creating infinite chains. The Zn^{2+} ion induced the ^{13}C NMR chemical shift changes of ligands (asn, gln, gln-m, and gln-e), as shown in Table 1.[29] Zn(II) complexes with gln and its derivatives indicate a downfield shift ($\Delta\delta = 4.5$–4.8) for carbonyl similar to that ($\Delta\delta = 4.7$) of the $Zn(asn)_2$ complex. The other carbons of Zn(II) complexes with gln and its derivatives also indicate similar shifts compared with those of $Zn(asn)_2$. These results reveal that the Zn^{2+} ion links exclusively to the carboxylic O atom and the α-amino N atom from each ligand, but the amide O atom does not behave so.

The Insulinomimetic Activity of Zn(II) Complexes. The in vitro insulinomimetic activity of Zn(II) complexes with gln derivatives was examined in relation to inhibiting the release of free fatty acid (FFA) from isolated rat adipocytes treated with epinephrine. For evaluating the insulinomimetic activity of compounds, we used the inhibition of FFA release from rat adipocytes.[26,30,31] The complexes were confirmed to act dose-dependently in concentrations of 10^{-4}, 5×10^{-4}, and 10^{-3} mol dm^{-3} of the Zn(II) complexes. The apparent IC_{50} values, and the 50% inhibitory concentration of the Zn(II) complexes upon FFA release, are given in Table 2. These Zn(II) complexes, with the exception of $Zn(gln)_2$, showed higher insulinomimetic activity than that of the standard $ZnSO_4$. Consequently, we concluded that the Zn(II) complexes with gln derivatives show higher insulinomimetic activities compared with those of the Zn^{2+} ion, such as $ZnSO_4$ and the complex, $Zn(gln)_2$. In previous research, we reported that Zn(II) complexes with amino acid derivatives, picolinic acid derivatives, and nicotine amide derivatives have high insulinomimetic activities.[13,18,24]

Table 2. Estimated IC_{50} (mM) Value for the Free Fatty Acids (FFA) Release from Isolated Rat Adipocytes of Zn(II) Complexes in the Presence of Glucose

Complex	IC_{50}/mM (± S.D.)[a)]	Complex	IC_{50}/mM (± S.D.)[a)]
$Zn(asn)_2$	0.80 (±0.04)[b)]	$Zn(gln)_2$	1.04 (±0.09)[b)]
$Zn(gln\text{-}m)_2$	0.61 (±0.07)[c)]	$Zn(gln\text{-}e)_2$	0.56 (±0.10)[c)]
$ZnSO_4$	1.00 (±0.12)		

a) Each value is expressed as the mean ± SD for 3 experiments. b) See Ref. 10. c) Significance at $p < 0.05$ vs $ZnSO_4$

Such Zn(II) complexes have been revealed to promote the glucose uptake into adipocytes, which in turn normalize the blood-glucose levels in experimental diabetic animals.[32] In this paper, following up on these compounds, we have now also confirmed insulinomimetic Zn(II) complexes with N-alkylated amides of gln, and, moreover, the Zn(II) complexes with derivatives of gln, which are the composition of green tea, could be possible candidates for anti-diabetic materials found in natural products.

Anti-diabetic Activity of Zn(II) Complex with Theanine (gln-e) on KK-A^y Mice. Based on these finding, we examined the in vivo anti-diabetic activity of a Zn(II) complex, $Zn(gln\text{-}e)_2$, on KK-A^y mice, and compared it with its ligand (gln-e).

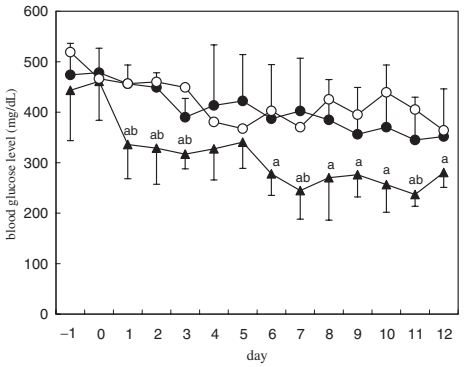

Fig. 1. Effect of $Zn(gln\text{-}e)_2$ on the glucose level of KK-A^y mice. Values are the mean ± SDs for the five mice in each group, control (○), gln-e treated (●), and $Zn(gln\text{-}e)_2$ treated (▲). (a) significance at $p < 0.05$ vs control KK-A^y mice. (b) significance at $p < 0.05$ vs KK-A^y mice treated with theanine.

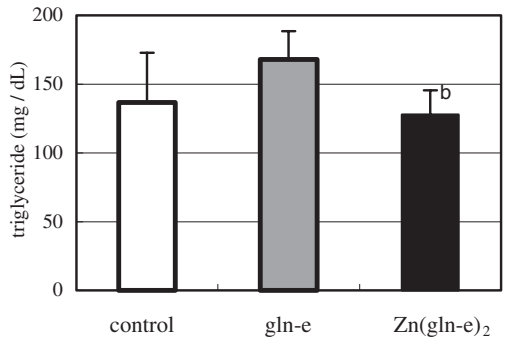

Fig. 2. Serum TG in control KK-A^y mice and KK-A^y mice receiving daily ip injection of gln-e and $Zn(gln\text{-}e)_2$ for 13 days. Values are the mean ± SDs for the five mice in each group. (a) significance at $p < 0.05$ vs control KK-A^y mice. (b) significance at $p < 0.05$ vs KK-A^y mice treated with gln-e.

During the treatment of $Zn(gln\text{-}e)_2$ or gln-e for about 2 weeks, the body weight of KK-A^y mice showed no differences between the three groups, including the control (data not shown). The blood-glucose level of KK-A^y mice (Fig. 1) was maintained under 300 mg/dL for 2 weeks in a $Zn(gln\text{-}e)_2$ treated group, which showed mostly a significant decrease compared to both the gln-e and control groups. On the other hand, it did not show any difference between the gln-e and control groups.

There were no differences in the serum BUN, ALT, AST, and TCHO levels between the control, treated with gln-e, and $Zn(gln\text{-}e)_2$ treated groups (data not shown).

However, the serum TG level, 127.7 ± 17.8 (mg/dL), in KK-A^y mice treated with $Zn(gln\text{-}e)_2$ decreased significantly compared to that, 168.0 ± 20.5, of the gln-e treated mice (Fig. 2).

There was no difference between the control and theanine group. The levels of HbA_{1c} are shown in Fig. 3.

HbA_{1c} analysis was used as an index to assess the glycemic control levels in DM. In the control KK-A^y mice and KK-A^y mice treated with gln-e, the HbA_{1c} levels before and after its

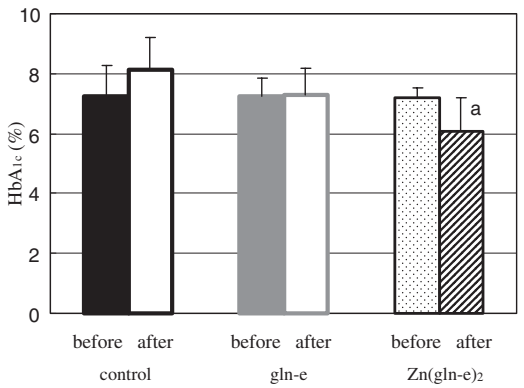

Fig. 3. Change in HbA_{1c} levels in KK-A^y mice before and after treatment with $Zn(gln\text{-}e)_2$ and gln-e. Values are the mean ± SDs for the five mice in each group. (a) significance at $p < 0.05$ vs pre in each group.

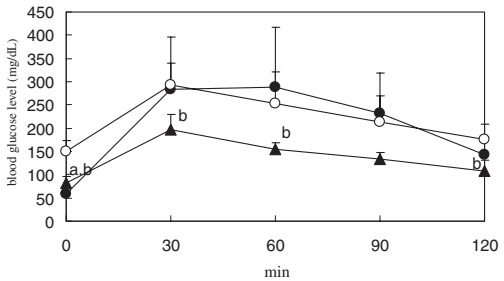

Fig. 4. Effects of $Zn(gln\text{-}e)_2$ on the oral glucose tolerance of KK-A^y mice. Oral glucose tolerance tests were performed on mice fasted for 16 h, then given the glucose solution orally at a dose of 1 g/kg body weight. Values are the mean ± SDs for the five mice in each group, control (○), gln-e treated (●), and $Zn(gln\text{-}e)_2$ treated (▲). (a) significance at $p < 0.05$ vs control KK-A^y mice. (b) significance at $p < 0.05$ vs KK-A^y mice treated with gln-e.

administration showed a tendency to increase from 7.24 ± 1.0 to 8.13 ± 1.1 (%) and 7.24 ± 0.6 to 7.28 ± 0.9 (%), respectively. In contrast, the HbA_{1c} level of the KK-A^y mice treated with $Zn(gln\text{-}e)_2$ decreased siginificantly before and after administration, 7.21 ± 1.0 to 6.1 ± 1.1 (%). These results suggest that $Zn(gln\text{-}e)_2$ sustained the longitudinal blood glucose controlling effect.

After 13 days of the administration of materials, the animals were given an oral glucose tolerance test (OGTT).

As shown in Fig. 4, the blood-glucose levels of the control group and the group treated with gln-e KK-A^y rose to the maximum concentration range of the Glucose tester, which was 300 mg/dL at 30 min after glucose administration, and then slowly decreased; there was no difference between both groups. In contrast, the blood glucose level of KK-A^y mice treated with $Zn(gln\text{-}e)_2$ also rose at first, to 197.2 ± 32.5 mg/dL, which was significantly lower compared with that of the control KK-A^y mice at each point in time of 0, 30, 60, and 120 min after administration.

These results indicate the possibility that the treatment of $Zn(gln\text{-}e)_2$ has a normalizing effect for blood glucose disorders on DM by improving glucose tolerance. Also, $Zn(gln\text{-}e)_2$ has

high insulinomimetic activity, as evaluated in vitro, and decreased the serum TG levels and HbA_{1c} concentrations in KK-A^y mice in vivo. Thus, Zn(gln-e)$_2$ could be a useful antidiabetic material. Recently, theanine (gln-e) has been suggested to reduce fat tissue in rodents,[33] and it is known that insulin secretion has a close relation to obesity.[34] Therefore, gln-e may strengthen the antidiabetic action of Zn(II) complex.

In conclusion, we propose new candidates of insulinomimetic Zn(II) complexes with N-alkylated amides of gln. In addition, the result of Zn(II) complexes with the compositions of Japanese green tea shows not only a high insulinomimetic activity in in-vitro experiments by the multiplication of zinc and the ligand, but also the possibility to develop novel, clinically useful and low toxic Zn(II) complexes from natural products. In particular, we can expect the theanine/Zn complex, Zn(gln-e)$_2$, to be a highly antidiabetic material.

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