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A [*meso*-Tetrakis(4-sulfonatophenyl)porphyrinato]zinc(II) Complex As an Oral Therapeutic for the Treatment of Type 2 Diabetic KKA^y Mice

Tapan K. Saha,*[a, b] Yutaka Yoshikawa,[b] and Hiromu Sakurai[b]

*We prepared and characterized [*meso*-tetrakis(4-sulfonatophenyl)porphyrinato]zinc(II) ([Zn(tpps)]), and investigated its in vitro insulin-mimetic activity and in vivo hypoglycemic effect in type 2 diabetic KKA^y mice. The results were compared with those of previously proposed insulin-mimetic zinc(II) complexes and zinc sulfate ($ZnSO_4$). The in vitro insulin-mimetic activity of [Zn(tpps)] was considerably better than that of bis(allixinato)zinc(II) ([Zn(alx)$_2$]), bis(maltolato)zinc(II) ([Zn(mal)$_2$]), bis(2-aminomethylpyridinato)zinc(II) ([Zn(2-ampy)$_2$]$^{2+}$), and $ZnSO_4$. In particular, the order of in vitro insulin-mimetic activity of the complexes was determined to be: [Zn(tpps)] > [Zn(alx)$_2$] > [Zn(mal)$_2$] > [Zn(2-ampy)]$^{2+}$ > $ZnSO_4$. [Zn(tpps)] normalized the hyperglycemia of KKA^y mice within 21 days when administered orally at doses of 10–20 mg (0.15–0.31 mmol) Zn per kg body mass for 28 days. In addition, metabolic syndromes such as insulin resistance, the degree of renal disturbance, and the degree of liver disturbance were significantly improved in [Zn(tpps)]-treated KKA^y mice relative to those administered with saline and $ZnSO_4$. The improvement in diabetes was validated by the results of oral glucose-tolerance tests and the decrease in the HbA_{1c} level observed. In contrast, $ZnSO_4$ and the ligand H_2tpps did not lower the elevated blood glucose level under the same experimental conditions. Based on these observations, [Zn(tpps)] is proposed to be the first orally active zinc(II)–porphyrin complex for the efficacious treatment of not only type 2 diabetes but also metabolic syndromes in animals.*

Introduction

Diabetes mellitus (DM) represents a heterogeneous group of disorders that have hyperglycemia as a common feature. Generally, DM can be divided into two main groups: one arises from the destruction of insulin-producing cells and the other results from a defect in insulin production.[1] These two main variants of DM, type 1 and type 2, differ in their pattern of inheritance, insulin response, and origin. Briefly, type 1 DM, which accounts for 5–10% of all cases, results from the destruction of β cells.[1] The major cause of islet cell destruction is autoimmunity (type 1A), and the minor cause is idiopathic (type 1B). About 80% of all DM cases are type 2. The two most important metabolic defects that characterize type 2 DM are aberrancies in β cell secretion of insulin and an inability of the peripheral target tissues to respond to insulin (insulin resistance). About 10% of DM cases are due to other reasons such as defective β cells, endocrine dysfunction, infection of the pancreas, chemical agents, and idiopathic causes. Most importantly, the long-term complications in blood vessels, kidneys, eyes, and nerves occurs in both types of DM and are the major cause of morbidity and death from DM.[2]

Depending on the type, the treatment of DM involves either daily injections of exogenous insulin (most common for type 1 DM) or oral administration of hypoglycemic drugs such as sulfonylureas, metformin, α-glucosidase inhibitors, thiazolidinediones and meglitinides, and combination therapies for type 2 DM.[3–6] However, this approach is not satisfactory for a large proportion of patients; hence, there have been continued efforts towards the development of new hypoglycemic drugs with high potency but with minor or no side effects.

In 1980, Coulston and Dandona[7] reported that zinc(II) chloride ($ZnCl_2$) stimulated lipogenesis in rat adipocytes similarly to the action of insulin. Following this finding, it has been established that zinc compounds exert an insulin-mimetic action in both in vitro and in vivo systems, including their ability to improve glucose homeostasis and insulin resistance in animal models of DM.[8–17]

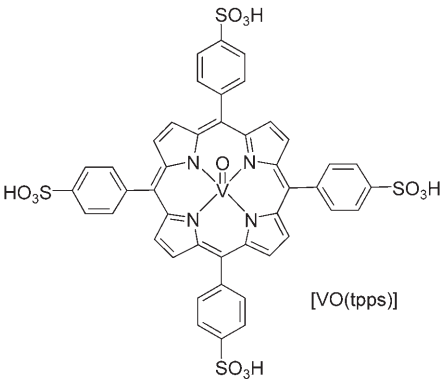
In recent years, several reports have documented zinc-therapy-induced improvements in controlling glucose metabolism and insulin sensitivity in the liver and muscles of many patients with type 1 and type 2 DM.[18–22] Moreover, Zn^{II} is an essential trace element in animals and humans.[23] It is a cofactor for >200 metalloenzymes that play a role in the maintenance of several cell functions. There is a strong relationship between

[a] *Dr. T. K. Saha*
Department of Chemistry, Colorado State University
Fort Collins, CO 80523 (USA)
Fax: (+1) 970-491-1801
E-mail: tksaha_ju@yahoo.com

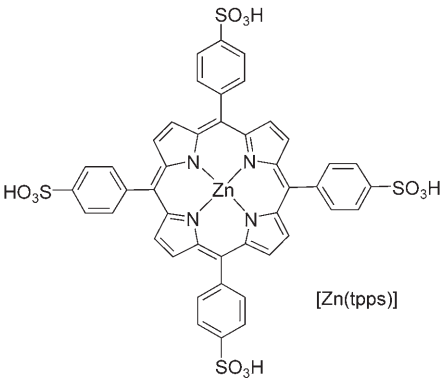
[b] *Dr. T. K. Saha, Dr. Y. Yoshikawa, Dr. H. Sakurai*
Department of Analytical and Bioinorganic Chemistry
Kyoto Pharmaceutical University
5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414 (Japan)

Zn^{II} and insulin, because each insulin hexamer contains two zinc ions.[24]

Since the discovery of the orally active insulin-mimetic bis(maltolato)zinc(II) ([Zn(mal)$_2$]) and bis(6-methylpicolinato)zinc(II) ([Zn(6-mpa)$_2$]) complexes for the treatment of type 2 DM rats (GK rats) in 2002,[16] the therapeutic potential of zinc(II) complexes has been of great interest to us. We have developed several types of zinc(II) complexes with different coordination spheres, such as Zn(N_2O_2) and Zn(O_4).[12,14,15,17] These complexes showed normoglycemic activity in type 2 DM animals. However, only a few complexes such as bis(2-aminomethylpyridinato)zinc(II) ([Zn(2-ampy)$_2$]$^{2+}$), bis(2-aminoethylpyridinato)zinc(II), ([Zn(2-aepy)$_2$]$^{2+}$), (1,5,8,12-tetraazadodecanato)zinc(II), ([Zn(1,5,8,12-td)]$^{2+}$), and (1,4,8,12-tetraazacyclopentadecanato)-zinc(II) ([Zn(15ane)]$^{2+}$), which have a Zn(N_4) zinc coordination sphere, have been examined.[25] Among these, [Zn(2-ampy)$_2$]$^{2+}$ was found to lower the blood glucose levels in KKA^y mice—a type 2 diabetic model—by intraperitoneal (ip) injections. However, this complex did not show blood glucose lowering activity in KKA^y mice by oral administration. Recently, we found that water-soluble [*meso*-tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadate(IV)$^{4-}$ ([VO(tpps)]), in which the vanadium coordination sphere is VO(N_4), is a potential insulin-mimetic oxovanadium(IV)–porphyrin complex for the treatment of not only KKA^y mice, but also streptozotocin (STZ)-induced diabetic mice—a type 1 diabetic model—when introduced by oral gavage.[26–29] This important finding prompted us to develop orally active zinc(II)–porphyrin complexes. Herein we report the complete synthesis of the [*meso*-tetrakis(4-sulfonatophenyl)porphyrinato]zinc(II) ([Zn(tpps)]) complex, its in vitro insulin-mimetic activity, and the in vivo normoglycemic and antidiabetic activities of [Zn(tpps)] upon oral administration to type 2 diabetic KKA^y mice for 28 days. Finally, we compare the observed results with those obtained by using zinc(II) sulfate ($ZnSO_4$) and previously reported insulin-mimetic zinc complexes as positive controls.



[VO(tpps)]



[Zn(tpps)]

Results and Discussion

Preparation and characterization of [Zn(tpps)]

[Zn(tpps)] was prepared according to the method used by Adler et al.[30] with slight modifications. The complex was characterized by elemental analysis, UV/Vis, and MS. The physicochemical parameters of [Zn(tpps)] are summarized in Table 1.

Table 1. Physicochemical properties of [Zn(tpps)].

Elemental Analysis ($C_{44}H_{28}O_{12}S_4N_4Zn\cdot17\,H_2O\cdot7\,C_3H_7NO$)	Calculated [%]	Found [%]
C	42.98	42.92
H	6.16	5.37
N	8.48	8.69
UV/Vis[a]		
	λ [nm]	ε [10^3 M^{-1} cm^{-1}]
λ_1	421	377.2
λ_2	557	23.8
λ_3	595	13.1

[a] Solvent = H_2O.

From the calculated and found values in the elemental analyses, DMF (7 mol) remained in the [Zn(tpps)] sample because of its high boiling point (150 °C). In the UV/Vis spectra of [Zn(tpps)] dissolved in H_2O, a Soret band at 421 nm and two visible bands at 557 and 595 nm were observed (spectrum not shown). The apparent molar absorptivity of [Zn(tpps)] was estimated to be 377.2×10^3, 23.8×10^3, and 13.1×10^3 M^{-1} cm^{-1} at 421, 557, and 595 nm, respectively, which are similar to the reported values.[31] The FAB(−) mass spectrum of [Zn(tpps)] had a molecular ion peak (*m/z*) at 997, which supports the elemental analysis data (Table 1). These results indicate that only mononuclear [Zn(tpps)] with a VO(N_4) coordination sphere is present in the examined sample solution.

In vitro insulin-mimetic activity of [Zn(tpps)]

The in vitro insulin-mimetic activity of the complexes was examined based on both the inhibition of free fatty acid release and the enhancement of glucose uptake in isolated rat adipocytes treated with epinephrine.[32,33] The concentration-dependent inhibitory effects of $ZnSO_4$ and [Zn(tpps)] on free fatty acid release in isolated rat adipocytes treated with epinephrine are illustrated in Figure 1a. The apparent IC_{50} values were estimated to be 0.440 ± 0.018 mM for $ZnSO_4$, 0.070 ± 0.003 mM for [Zn(tpps)], 0.323 ± 0.127 mM for [Zn(2-ampy)]$^{2+}$, 0.369 ± 0.145 mM for [Zn(1,5,8,12-td)]$^{2+}$, 0.151 ± 0.011 mM for bis(allixi-

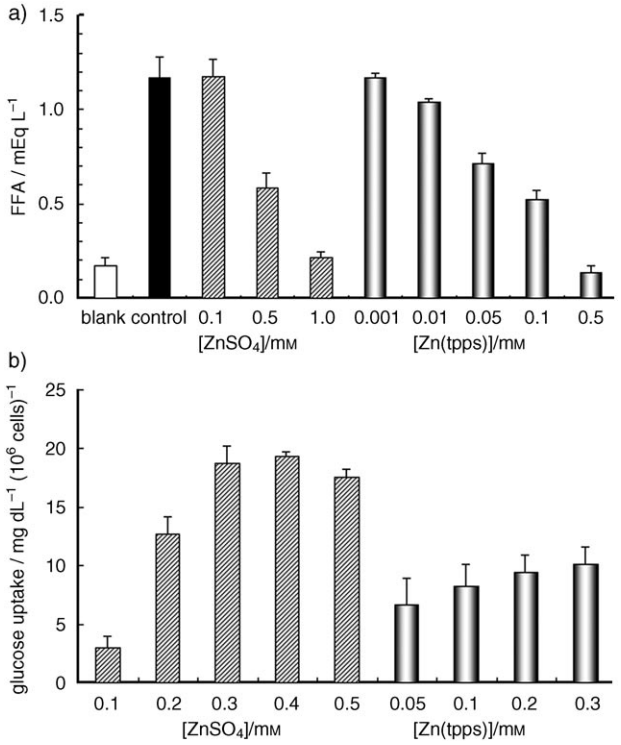


Figure 1. Inhibitory effects of $ZnSO_4$ and [Zn(tpps)] on a) free fatty acid (FFA) release and b) their enhancing effect on glucose uptake in rat isolated adipocytes (2.5×10^6 cells mL^{-1}) treated with 0.01 mM epinephrine in the presence of 1 mg mL^{-1} glucose.

nato)zinc(II) ([Zn(alx)$_2$]), and 0.220 ± 0.028 mM for [Zn(mal)$_2$] (Table 2).[25,34] In particular, [Zn(tpps)] exhibited the highest inhibitory activity among all of the complexes studied, whereby the order of inhibitory activity of the complexes is: [Zn(tpps)] > [Zn(alx)$_2$] > [Zn(mal)$_2$] > [Zn(2-ampy)]$^{2+}$ > [Zn(1,5,8,12-td)]$^{2+}$ > $ZnSO_4$.

$ZnSO_4$ and [Zn(tpps)] also produced a concentration-dependent increase in glucose uptake in epinephrine-treated isolated rat adipocytes (Figure 1b). The apparent EC_{50} values were estimated to be 0.176 ± 0.009 mM for $ZnSO_4$, 0.088 ± 0.029 mM for [Zn(tpps)], 0.139 ± 0.005 mM for [Zn(alx)$_2$], and 0.237 ± 0.007 mM for [Zn(mal)$_2$] (Table 2).[17] The apparent EC_{50} values of the zinc complexes suggest that [Zn(tpps)] has the highest enhancing activity among all of the zinc complexes studied. Based on these results, [Zn(tpps)] is anticipated to have a higher in vitro insulin-mimetic activity than those of $ZnSO_4$ and other zinc complexes reported previously.[17,25,34]

Table 2. Apparent IC_{50} and EC_{50} values for $ZnSO_4$ and zinc complexes in isolated rat adipocytes treated with epinephrine.

Complex	FAA[a] IC_{50} [mM]	Glucose Uptake EC_{50} [mM]
$ZnSO_4$	0.440 ± 0.018	0.176 ± 0.009
[Zn(tpps)]	0.070 ± 0.003[b]	0.088 ± 0.029[b]
[Zn(2-ampy)]$^{2+}$	0.323 ± 0.127[25]	not measured
[Zn(1,5,8,12-td)]$^{2+}$	0.369 ± 0.145[25]	not measured
[Zn(alx)$_2$]	0.151 ± 0.011[34]	0.139 ± 0.005[17]
[Zn(mal)$_2$]	0.220 ± 0.028[34]	0.237 ± 0.007[17]

[a] Free fatty acid assay. [b] Significance at $p<0.01$ versus $ZnSO_4$ (Student *t* test).

In vivo evaluation of antidiabetic activity of [Zn(tpps)] in KKA^y mice

We examined the blood glucose lowering effects of [Zn(tpps)], which showed the highest in vitro insulin-mimetic activity, in KKA^y mice upon oral administration for 28 days and compared them with those of the control agent $ZnSO_4$. Figure 2 shows the change in blood glucose levels in the saline-treated nondiabetic C57BL/6J mice (Group I) and KKA^y mice treated with saline alone (Group II), $ZnSO_4$ in saline (Group III), and [Zn(tpps)] in saline (Group IV) at doses in the range of 10–20 mg (0.15–0.31 mmol) Zn per kg body mass, as well as KKA^y mice treated with the ligand *meso*-tetrakis(4-sulfonatophenyl)porphyrin (H_2tpps) in saline (Group V) at doses corresponding to the equimolar concentration of [Zn(tpps)]. The blood glucose concentration in the saline-treated KKA^y mice (Group II) was significantly higher than that of the saline-treated nondiabetic C57BL/6J mice (Group I) throughout the study. Administration of $ZnSO_4$ at a dose of 10 mg (0.15 mmol) Zn per kg body mass for the first 5 days did not result in a decreased blood glucose level in the KKA^y mice from Group III. An increase in the dose to 20 mg (0.31 mmol) Zn per kg body mass for the following 23 days also did not result in a lower blood glucose level in comparison with that of saline-treated KKA^y mice. In contrast, administration of [Zn(tpps)] at a dose of 10 mg (0.15 mM) Zn per kg body mass resulted in a rapid decrease in the blood glucose level of KKA^y mice (Group IV) after 1 day. The same dosage of the complex was then maintained for the following 4 days. However, the blood glucose levels increased again, and then the administration dose was increased to 20 mg

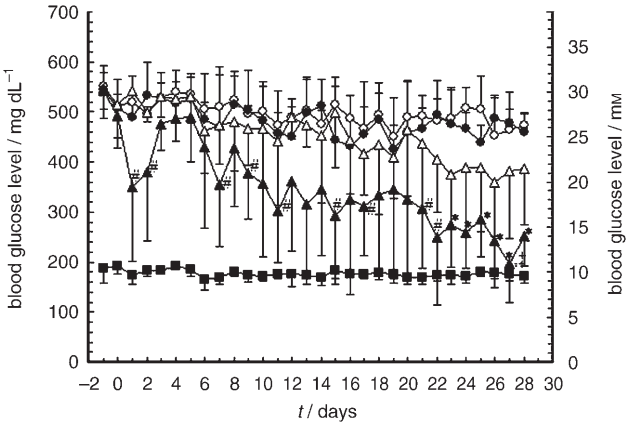


Figure 2. Changes in blood glucose levels in saline-treated nondiabetic CB57/6J mice (■, $n=5$) and type 2 diabetic KKA^y mice after oral administration for 28 days of saline (○, $n=5$), $ZnSO_4$ (△, $n=5$), and [Zn(tpps)] (▲, $n=5$) at doses of 10–20 mg (0.15–0.31 mmol) Zn per kg body mass, and H_2tpps (●, $n=5$) at doses corresponding to the equimolar concentration of [Zn(tpps)]. $ZnSO_4$, [Zn(tpps)], and H_2tpps were dissolved in saline. Levels of significance: $*p<0.01$ and $\#p<0.05$ versus saline-treated KKA^y mice; $+p<0.05$ versus $ZnSO_4$-treated KKA^y mice.

(0.31 mmol) Zn per kg body mass for the following 23 days. After such adjustment, the blood glucose level gradually lowered and remained at approximately 250 mg dL^{-1} (10 mM) for the last 7 days; this value was close to the blood glucose levels in the nondiabetic C57BL/6J mice (Figure 2). There was no significant difference between the blood glucose level of the [Zn(tpps)]-treated KKA^y mice and the C57BL/6J mice for the last 7 days. Similar phenomena were also observed in the case of [VO(tpps)]-treated KKA^y mice.[26] However, H_2tpps did not show any hypoglycemic effects at doses equivalent to those of [Zn(tpps)] (Figure 2). Reportedly, the blood glucose level remained around 300 mg dL^{-1} (16.7 mM) in KKA^y mice after oral treatment with $[Zn(alx)_2]$ and bis(L-carnitinato)zinc(II) ($[Zn(car)_2]Cl_2$) suspensions in 5% acacia at doses of 15–20 mg (0.23–0.31 mmol) Zn per kg body mass for 14–16 days.[34,35] These results demonstrate that following chronic oral gavage, [Zn(tpps)] has a higher normoglycemic activity in KKA^y mice than $ZnSO_4$, $[Zn(alx)_2]$, and $[Zn(car)_2]Cl_2$. Based on these results, [Zn(tpps)] is anticipated to be able to normalize the elevated blood glucose levels in type 2 diabetic KKA^y mice.

Changes in body mass and in food and water intake

Daily changes in body mass in each group of mice are shown in Figure 3. There were no significant changes between the initial and final body mass of the CB57/6J and KKA^y mice used in this study. The initial body mass was similar in the four groups of KKA^y mice (41.3 ± 3.5 g, saline-treated group; 42.4 ± 1.2 g, $ZnSO_4$-treated group; 40.9 ± 3.6 g, [Zn(tpps)]-treated group; 42.2 ± 3.2 g, H_2tpps-treated group). However, body mass tended to be low in the nondiabetic CB57/6J mice (26.2 ± 1.5 g). After 28 days of treatment, gain in body mass was similar in all groups (KKA^y mice: 42.6 ± 2.7 g, saline-treated group; 42.5 ± 1.1 g, $ZnSO_4$-treated group; 41.2 ± 2.8 g, [Zn(tpps)]-treated group; 43.7 ± 2.0 g, H_2tpps-treated group and CB57/6J mice: 27.7 ± 1.8 g, saline-treated group).

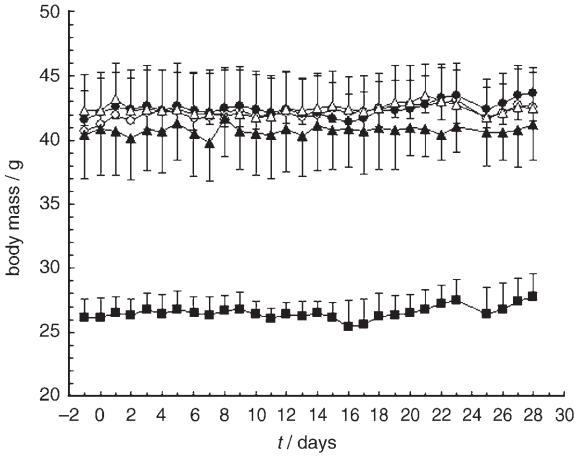


Figure 3. Changes in body mass of saline-treated nondiabetic CB57/6J mice (■, $n=5$) and type 2 diabetic KKA^y mice after oral administration for 28 days of saline (○, $n=5$), $ZnSO_4$ (△, $n=5$), and [Zn(tpps)] (▲, $n=5$) at doses of 10–20 mg (0.15–0.31 mmol) Zn per kg body mass, and H_2tpps (●, $n=5$) at doses corresponding to the equimolar concentration of [Zn(tpps)]. $ZnSO_4$, [Zn(tpps)], and H_2tpps were dissolved in saline.

Food and water intake per mouse in each group was measured daily throughout the experiment. The daily food intake in the saline-treated KKA^y mice was significantly higher than that in the CB57/6J mice (Figure 4a), indicating a high urinary clearance of glucose in the KKA^y mice. In contrast, the daily food intake was low in the $ZnSO_4$- and [Zn(tpps)]-treated mice compared with that in the saline-treated KKA^y mice after 1 or 2 days, and remained constant throughout the experiment. Moreover, there was no statistically significant difference in the daily food consumption observed in the CB57/6J mice and the KKA^y mice treated with [Zn(tpps)] (Figure 4a), suggesting that food intake is correlated with suppression of the urinary clearance of glucose.

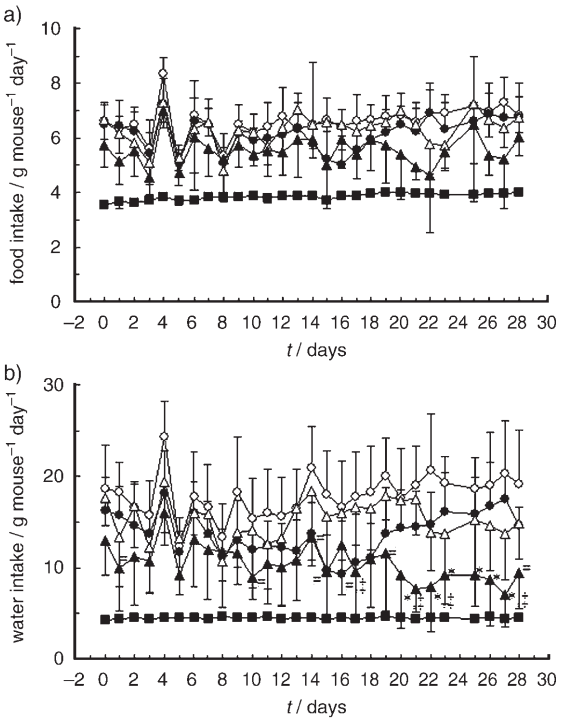


Figure 4. Changes in a) food intake and b) water intake in saline-treated nondiabetic CB57/6J mice (■, $n=5$) and type 2 diabetic KKA^y mice after oral administration for 28 days of saline (○, $n=5$), $ZnSO_4$ (△, $n=5$), and [Zn(tpps)] (▲, $n=5$) at doses of 10–20 mg (0.15–0.31 mmol) Zn per kg body mass, and H_2tpps (●, $n=5$) at doses corresponding to the equimolar concentration of [Zn(tpps)]. $ZnSO_4$, [Zn(tpps)], and H_2tpps were dissolved in saline. Levels of significance: $*p<0.01$ and $\#p<0.05$ versus saline-treated KKA^y mice; $\mp p<0.05$ versus $ZnSO_4$-treated KKA^y mice.

Water intake in the $ZnSO_4$- and [Zn(tpps)]-treated mice was significantly decreased relative to that of the saline-treated KKA^y mice after 1 day and remained almost constant throughout the experiment (Figure 4b). Moreover, water intake in the CB57/6J mice and the [Zn(tpps)]-treated KKA^y mice was almost the same after 1 day. As the administration of the [Zn(tpps)] complex to type 2 diabetic KKA^y mice resulted in a lowering of the blood glucose level without causing any loss of body mass in the animals, it is assumed that hyperglycemia was not modi-

fied by food uptake, but was controlled by [Zn(tpps)] treatment.

Change in the HbA_{1c} level

After the 28 days of treatment, we examined the change in glycosylated hemoglobin A_{1c} (HbA_{1c}) levels in the C57BL/6J mice and the four groups of KKA^y mice (Figure 5). The rate of

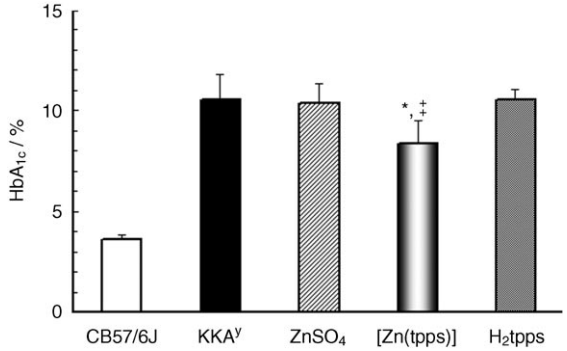


Figure 5. Changes in HbA_{1c} levels in saline-treated nondiabetic CB57/6J mice ($n=5$) and type 2 diabetic KKA^y mice after oral administration for 28 days of saline ($n=5$), $ZnSO_4$ ($n=5$), and [Zn(tpps)] ($n=5$) at doses of 10–20 mg (0.15–0.31 mmol) Zn per kg body mass, and H_2tpps ($n=5$) at doses corresponding to the equimolar concentration of [Zn(tpps)]. $ZnSO_4$, [Zn(tpps)], and H_2tpps were dissolved in saline. Levels of significance: $*p<0.01$ versus saline-treated KKA^y mice; $\ddagger p<0.05$ versus $ZnSO_4$-treated KKA^y mice.

HbA_{1c} formation is directly proportional to the ambient glucose concentration. Because erythrocytes are freely permeable to glucose, the level of HbA_{1c} in a blood sample from humans provides a glycemic history of the previous 120 days—the average erythrocyte lifespan. The change in HbA_{1c} levels has been used as an index of glycemic control for both research purposes and in diabetic patients.[36,37] The HbA_{1c} level ($8.4\pm1.2\%$) in the [Zn(tpps)]-treated KKA^y mice decreased significantly ($p<0.01$) compared with that in the saline-treated ($10.5\pm1.3\%$) KKA^y mice. Similarly, the HbA_{1c} level in the [VO(tpps)]-KKA^y mice was found to be $5.5\pm1.2\%$.[26] In contrast, $ZnSO_4$ and H_2tpps did not improve the HbA_{1c} levels in the KKA^y mice (Figure 5). These results indicate that [Zn(tpps)] treatment achieves good glycemic control in KKA^y mice, although the HbA_{1c} level of the C57BL/6J mice was estimated to be $3.6\pm0.2\%$.

Oral glucose tolerance test

To evaluate the improvement in glucose tolerance in KKA^y mice, an oral glucose tolerance test (OGTT) was performed on all experimental mice after the 28 days of treatment. As shown in Figure 6, after oral administration of glucose at a dose of 1 g kg^{-1} body mass, the blood glucose levels of the CB57/6J mice increased to a maximal concentration of 245 mg dL^{-1} (13.6 mM) after 15 min and then decreased sharply. In contrast, the blood glucose level of saline-treated KKA^y mice increased to a maximal concentration of 374 mg dL^{-1} (21 mM) after 15 min and then decreased gradually. Similar phenomena were

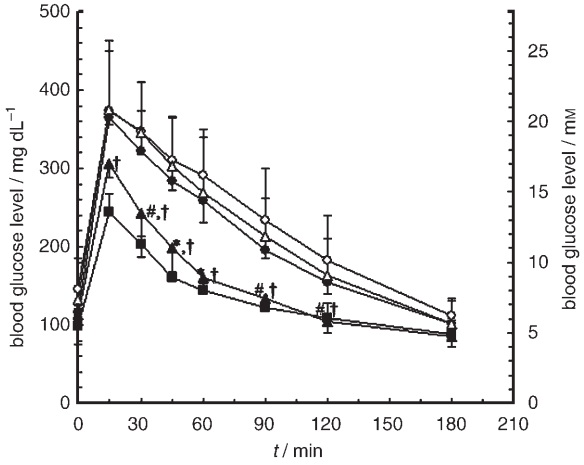


Figure 6. Oral glucose tolerance tests for saline-treated nondiabetic CB57/6J mice (■, $n=5$) and type 2 diabetic KKA^y mice after 28 days of oral administration of saline (○, $n=5$), $ZnSO_4$ (△, $n=5$), and [Zn(tpps)] (▲, $n=5$) at doses of 10–20 mg (0.15–0.31 mmol) Zn per kg body mass, and H_2tpps (●, $n=5$) at doses corresponding to the equimolar concentration of [Zn(tpps)]. $ZnSO_4$, [Zn(tpps)], and H_2tpps were dissolved in saline. Oral glucose tolerance tests were performed on mice that had fasted for 12 h. Afterward they were administered an oral glucose solution at a dose of 1 g kg^{-1} body mass. Each symbol is expressed as the mean $\pm$SD ($n=5$). Levels of significance: $*p<0.01$ and $\#p<0.05$ versus saline-treated KKA^y mice; $\dagger p<0.01$ versus $ZnSO_4$-treated KKA^y mice.

observed in the cases of $ZnSO_4$- and H_2tpps-treated KKA^y mice (Figure 6). However, the elevation of the blood glucose level in KKA^y mice treated with [Zn(tpps)] was significantly suppressed (305 mg dL^{-1} or 17 mM after 15 min) relative to that of the saline-treated (374 mg dL^{-1} or 19 mM after 15 min) and $ZnSO_4$-treated (375 mg dL^{-1} or 21 mM after 15 min) KKA^y mice. These results demonstrate that the impaired glucose tolerance in KKA^y mice is ameliorated by [Zn(tpps)] treatment, as was observed with [VO(tpps)].[26]

Serum parameters

The levels of urea nitrogen (UN), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), triglycerides (TG), total cholesterol (TCHO), and free fatty acids (FFA) in the serum of the C57BL/6J and KKA^y mice are summarized in Table 3. The serum UN level (26.0 ± 1.7 mg dL^{-1}), which indicates the degree of renal disturbance, was lowered significantly in the [Zn(tpps)]-treated KKA^y mice compared with that of the saline-treated (32.3 ± 3.9 mg dL^{-1}; $p<0.01$) and $ZnSO_4$-treated (29.3 ± 4.3 mg dL^{-1}; $p<0.05$) KKA^y mice. Moreover, the serum UN level in the CB57/6J mice was estimated to be 25.9 ± 1.9 mg dL^{-1}. The GPT level (16 ± 3 U L^{-1}) in the [Zn(tpps)]-treated KKA^y mice, which shows the degree of liver disturbance, was also significantly suppressed compared with those of saline-treated KKA^y mice, although the serum GOT levels were same in both cases (Table 3). The serum GOT and GPT levels in the CB57/6J mice were 66 ± 6 and 16 ± 2 U L^{-1}, respectively. On the other hand, the serum TCHO, TG, and FFA levels in [Zn(tpps)]-treated KKA^y mice were not altered relative to those of the saline-treated KKA^y mice (Table 3). Moreover,

Table 3. Serum parameters in saline-treated nondiabetic CB57/6J and KKA^y mice treated with saline alone, $ZnSO_4$, [Zn(tpps)], and H_2tpps in saline after daily oral administrations for 28 days.

Sample	Dose [mg Zn kg^{-1}] ([mmol])	*n*	UN [mg dL^{-1}]	GOT [U L^{-1}]	GPT [U L^{-1}]	TG [mg dL^{-1}]	TCHO [mg dL^{-1}]	FFA [mEq L^{-1}]	Insulin [μU mL^{-1}]
Saline-treated CB57/6J mice		5	25.9 ± 1.9	66 ± 6	16 ± 2	85 ± 8	103 ± 13	0.904 ± 0.101	7.70 ± 1.91
Saline-treated KKA^y mice		5	32.3 ± 3.9	67 ± 9	22 ± 3	220 ± 69	154 ± 13	1.725 ± 0.151	26.62 ± 8.69
$ZnSO_4$-treated KKA^y mice	10–20 (0.15–0.31)	5	29.3 ± 4.0	63 ± 7	22 ± 4	214 ± 25	177 ± 31	1.682 ± 0.167	25.09 ± 5.30
[Zn(tpps)]-treated KKA^y mice	10–20 (0.15–0.31)	5	26.0 ± 1.7[a,b]	68 ± 8	16 ± 3[a,b]	209 ± 37	156 ± 8	1.473 ± 0.169[c]	8.08 ± 1.94[a,d]
H_2tpps-treated KKA^y mice	(0.15–0.31)	5	27.8 ± 3.4	67 ± 13	22 ± 8	232 ± 50	161 ± 15	1.628 ± 0.347	22.62 ± 9.00

Levels of significance: [a] $p < 0.01$ and [b] $p < 0.05$ versus KKA^y mice given saline only; [d] $p < 0.01$ and [c] $p < 0.05$ versus KKA^y mice treated with $ZnSO_4$.

the serum UN, GPT, GOT, TG, and TCHO levels in the KKA^y mice treated with either saline alone or with $ZnSO_4$ and H_2tpps dissolved in saline were quite similar, indicating that no serum parameters in KKA^y mice are improved by $ZnSO_4$ and H_2tpps.

The serum insulin level (8.1 ± 1.9 μU mL^{-1}) in the [Zn(tpps)]-treated KKA^y mice was decreased significantly relative to that of saline-treated KKA^y mice (Table 3). It is well known that KKA^y mice show high insulinemia and develop DM.[38,39] Serum insulin was also estimated to be 7.7 ± 1.9 μU mL^{-1} in the CB57/6J mice and 25.1 ± 5.3 μU mL^{-1} and 22.6 ± 5.6 μU mL^{-1} in the $ZnSO_4$- and H_2tpps-treated KKA^y mice, respectively (Table 3). These findings demonstrate that [Zn(tpps)] and [VO(tpps)][26] have antidiabetic potency through not only their high blood glucose-lowering effect but also as a result of their ability to attenuate the insulin resistance in type 2 diabetic KKA^y mice.

To evaluate the mode of action of the insulin-mimetic activity of zinc compounds, we examined the insulin-mimetic activity of $ZnSO_4$, bis(picolinato)zinc(II) ([$Zn(pa)_2$]), bis(threoninato)-zinc(II) ([$Zn(threonin)_2$]), and [$Zn(mal)_2$] in isolated rat adipocytes using four different inhibitors of glucose and fatty acid metabolism: cytochalasin B (a glucose transporter 4 (GLUT4) inhibitor), cilostamide (a phosphodiesterase inhibitor), HNMPA-(AM_3) (a tyrosine kinase inhibitor), and wortmannin (a phosphoinositide-3 kinase (PI3-k) inhibitor).[40] We confirmed that the zinc(II) ion and its complexes promote glucose uptake into adipocytes by affecting at least three sites in these cells, which in turn normalize blood glucose levels in the experimental diabetic animals. Therefore, we speculate that [Zn(tpps)] acts in a similar manner to those complexes. However, there is still a need for a detailed investigation on the mechanism of action responsible for the insulin-mimetic activity of [Zn(tpps)] in type 2 DM mice.

In conclusion, [Zn(tpps)] is the first example of an orally active zinc(II)–porphyrin complex with a $Zn(N_4)$ coordination environment that is useful for treating type 2 diabetic KKA^y mice and their metabolic syndromes.

Experimental Section

Materials: All reagents and solvents were obtained from commercially available sources and were of the highest purity grade; hence, they were used without purification. Zinc(II) sulfate, $ZnSO_4 \cdot 7H_2O$, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The ligand *meso*-tetrakis(4-sulfonatophenyl)porphyrin (H_2tpps) was purchased from Frontier Scientific Inc. (Logan, UT, USA). Sephadex LH-20 was obtained from Amersham Pharmacia Biotech (Tokyo, Japan). Bovine serum albumin (fraction V), (±)-epinephrine monohydrochloride, and collagenase were purchased from Sigma Chemical (St. Louis, MO, USA).

Animals: Male nondiabetic C57BL/6J mice (5 weeks old, 17–19 g) and male KKA^y mice (5 weeks old, 25–27 g) were obtained from CLEA Japan, Inc. (Osaka, Japan), and were used for in vivo study when they were 12 weeks old. KKA^y mice, which are obtained by crossing glucose-intolerant black KK female mice and yellow male obese A^y mice, are characterized by hyperphagia due to leptin resistance, followed by obesity, and the development of hyperleptinemia, insulin resistance, hyperinsulinemia, diabetes, dyslipidemia, and hypertension after approximately 8 weeks of age.[41–43] Therefore, KKA^y mice are known to serve as an excellent model that closely resembles obesity-linked type 2 diabetes in humans who express several disorders within a single individual. The C57BL/6J mice were generally employed as a nondiabetic control for the KKA^y mice. All animals were individually housed in a cage and maintained on a light–dark cycle of 12 h in a temperature-controlled central animal room. All mice were allowed free access to solid food (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water. All animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and performed according to the guidelines for animal experimentation developed by KPU.

Synthesis of [Zn(tpps)] complex: The zinc(II) derivative of H_2tpps was prepared according to the method used by Adler et al.[30] with slight modifications. H_2tpps (2.5 g, 2.48 mmol) and zinc(II) sulfate ($ZnSO_4 \cdot 7H_2O$, 1.43 g, 4.96 mmol) were heated at reflux (160 °C) in *N*,*N*-dimethylformamide (DMF, 250 mL) while stirring for 8 h. The solution was then evaporated to approximately 5 mL and cooled in an ice bath. Acetone (20 mL) was added to the remaining solution. The resulting precipitate was redissolved in methanol and reprecipitated with acetone (6×). The crude material was purified by gel chromatography (Sephadex LH-20; eluent: methanol). Finally, the resulting solution was concentrated and dried under high vacuum. The composition of the complex was determined by elemental analyses, MS and UV/Vis (Table 1), and then compared with the data from relevant literature.[31]

Physical measurements: Elemental analyses were carried out using a PerkinElmer 240C elemental analyzer (Wellesley, MA, USA). Low-resolution mass spectra were recorded with a JEOL JMS-SX 102AQQ spectrometer (JEOL, Tokyo, Japan) in FAB(−) mode by using methanol or 3-nitrobenzyl alcohol as the matrix material; this was performed at the Analytical Center of Kyoto Pharmaceuti-

cal University. UV/Vis absorption spectra in aqueous solvents were recorded with an Agilent 8453 UV/Vis spectrometer (Agilent, Germany).

Evaluation of in vitro insulin-mimetic activity: The insulin-mimetic activity of [Zn(tpps)] was evaluated by simultaneous in vitro experiments in which both the inhibitory activity upon release of free fatty acid[32] and the enhancement of glucose uptake[33] of the complex in isolated rat adipocytes treated with epinephrine were estimated and compared with those of $ZnSO_4$ as a positive control. Male Wistar rats (200 g) were sacrificed under anesthesia with ether. The adipose tissues were removed, chopped with scissors, and digested with collagenase for 1 h at 37 °C in Krebs Ringer bicarbonate buffer (pH 7.4) containing 2% bovine serum albumin. The adipocytes thus obtained were then separated from undigested tissues by filtration through a nylon mesh (250 μm) and washed three times with the buffer in the absence of collagenase. The complex was dissolved in saline at various concentrations. 30 μL of each solution and 10 μL glucose (final concentration: 5 mM) along with 5 μL H_2O were added to 240 μL of the isolated adipocytes (1×10^6 cells mL^{-1}), and the resulting suspensions were incubated at 37 °C for 30 min. Finally, 15 μL epinephrine (final concentration: 10 μM) was added to the suspensions, and the resulting mixture was incubated at 37 °C for 3 h. The reaction was stopped by cooling in ice water, and the mixtures were centrifuged at 3000 rpm at 4 °C for 10 min. The free fatty acid concentration in the extracellular solution was determined with a free fatty acid kit (NEFA C-test Wako, Wako Pure Chemical Industries, Osaka, Japan). The IC_{50} value (50% inhibitory concentration of the complex) was determined from the curve for the concentration-dependent inhibitory effect of the complex on free fatty acid release in isolated rat adipocytes treated with epinephrine. In addition, the glucose concentration in the extracellular solution was estimated using a Fuji Dry Chem analyzer (Fuji Medical Co., Tokyo, Japan). The glucose uptake of the compounds was evaluated using the apparent EC_{50} values, which are defined as the 50% enhancing concentration of the compound with respect to the maximal glucose uptake concentration in epinephrine-treated adipocytes.

Evaluation of in vivo antidiabetic activity in KKA^y mice: Mice were divided into the following five groups: Group I, nondiabetic C57BL/6J mice ($n=5$) that were orally administered saline alone; Group II, control KKA^y mice ($n=5$) that were orally administered saline alone; Group III, KKA^y mice ($n=5$) that were orally administered $ZnSO_4$ dissolved in saline; Group IV, KKA^y mice ($n=5$) that were orally administered [Zn(tpps)] dissolved in saline; and Group V, KKA^y mice ($n=5$) that were orally administered H_2tpps dissolved in saline. $ZnSO_4$ and [Zn(tpps)] dissolved in saline were administered to the mice from Groups III and IV, respectively, at doses of 10 mg (0.15 mmol) Zn per kg body mass for the first 5 days and then 20 mg (0.31 mmol) Zn per kg body mass for the following 23 days. The ligand H_2tpps dissolved in saline was administered to the mice from Group V at doses corresponding to the equimolar concentration of [Zn(tpps)]. These agents were administered to each mouse for 28 days at about 11:00 AM after the determination of their blood glucose level. The blood sample required for the daily analyses of glucose levels was collected from the tail vein of each mouse, and the blood glucose level was measured using a GLUCOCARD (ARKRAY Inc., Kyoto, Japan). The body mass of the mice was measured daily before the administration of the saline and Zn^{II} compounds. Moreover, the intake of solid food and drinking water by each mouse was ensured daily throughout the experiment before the administration of the saline and Zn^{II} compounds.

After the oral administration of saline alone and $ZnSO_4$, [Zn(tpps)], and H_2tpps dissolved in saline for 28 days, blood samples were collected by orbital exsanguination from the mice anesthetized with ether and were centrifuged at 5000 rpm for 10 min at 4 °C. The serum samples were separated and subjected to the analyses of urea nitrogen (UN), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), triglycerides (TG), total cholesterol (TCHO), free fatty acids (FFA), and insulin levels. The serum UN, GPT, GOT, TG, and TCHO levels were estimated by using a FUJI DRY-CHEM analyzer (FUJIFILM Medical Co. Ltd., Tokyo, Japan). The NEFA C-test and Glazyme insulin-EIA test (Wako Pure Chemical Industries Ltd., Osaka, Japan) were used to determine the serum FFA and insulin levels, respectively. Moreover, the glycosylated hemoglobin (HbA_{1c}) levels in the blood collected from the tail veins of the mice were measured using a DCA 2000 analyzer (Bayer Corp., Tokyo, Japan).

Oral glucose tolerance test: After the administration of saline alone and $ZnSO_4$, [Zn(tpps)], and H_2tpps dissolved in saline for 28 days, oral glucose tolerance tests (OGTT) were performed. All mice were fasted for 12 h, and glucose at a dose of 1 g kg^{-1} body mass was administered orally. Blood samples were collected from the tail veins at 0, 15, 30, 45, 60, 90, 120, and 180 min after glucose administration. The blood glucose levels were measured using the GLUCOCARD as described above.

Statistical analysis: All experimental results are expressed as mean ± SD. Statistical analysis was performed by using analysis of variance (ANOVA) or Student *t* test at 5% ($p<0.05$) or 1% ($p<0.01$) levels of significance.

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