

Cellular Mechanism of Zinc–Hinokitiol Complexes in Diabetes Mellitus

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Zinc complexes exhibit high insulin-like and antidiabetic activities in animals with type 2 diabetes mellitus (DM). However, molecular mechanisms underlying these activities have not yet been determined. In this study, we investigated activation of the insulin signaling pathway by Zn in 3T3-L1 adipocytes using di(hinokitiolato)zinc complex (hinokitiol: 2-hydroxy-4-isopropylcyclohepta-2,4,6-trienone, $[Zn(hnk)_2]$), which exhibits antidiabetic effect in animals with type 2 DM. Our results show that $[Zn(hnk)_2]$ strongly induced Akt/protein kinase B (Akt/PKB) phosphorylation, and optimal phosphorylation was achieved at a concentration of 50 μM. The $[Zn(hnk)_2]$-induced Akt/PKB phosphorylation was almost completely inhibited by wortmannin, whereas $[Zn(hnk)_2]$-induced phosphorylation of glycogen synthase kinase-3β (GSK3β) was partially inhibited by wortmannin. Further, we examined cellular Zn uptake by $[Zn(hnk)_2]$ stimulation and evaluated cellular glucose uptake at the same time point. The intracellular Zn concentration incubated with $[Zn(hnk)_2]$ was approximately 4.7-fold higher than that in the control cells. Moreover, the glucose uptake of $[Zn(hnk)_2]$-treated adipocytes was 3.7-fold higher than that of the control adipocytes. These results suggest that $[Zn(hnk)_2]$ was able to translocate glucose transporter 4 (GLUT4) protein to the plasma membrane. Thus, we propose that $[Zn(hnk)_2]$ produces the antidiabetic effect by inducing insulin signaling pathways and glucose uptake.

Diabetes mellitus (DM) includes a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion or function.[1] In 2030, the worldwide prevalence of DM in all age groups is expected to rise to approximately 4.4% of the world population or 366 million people.[2] DM with complications, such as diabetic nephropathy, retinopathy, and neuropathy, is difficult to treat. Therefore, early treatment of DM is extremely important. On the basis of its etiology, DM is classified into 2 main types: type 1 DM and type 2 DM. Type 1 DM is an autoimmune disease characterized by β-cell destruction and insulin injections are administered for its management, whereas type 2 DM refers to adult-onset DM caused by defective insulin sensitivity, managed by the administration of several types of synthetic drugs.[3] For DM treatment, some patients use several medicines, which cause severe side effects in the long-term. Other patients may experience pain and high stress levels due to multiple insulin injections administered on a daily basis. Therefore, development of new compounds without severe side effects for type 2 DM is crucial not only for treating DM but also for improving DM patients' quality of life.

Zinc is an essential trace element with multiple regulatory functions, involving insulin synthesis, insulin secretion, and signaling.[4] Zn plays important roles in cellular homeostasis, e.g., Zn deficiency and Zn overload have been reported to disrupt signal transduction pathways involved in growth factor receptor signaling, eventually altering cell proliferation, differentiation, and survival, leading to apoptosis.[5,6] For many years, insulin-like effects of Zn ions have been reported in rat adipocytes. It has been demonstrated that Zn increases lipogenesis[7] and stimulates glucose transport by receptor- and kinase-mediated mechanisms.[8] Later, Tang et al. reported that insulin-like effects of Zn ions on glucose transport are mediated by phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (Akt/PKB) in 3T3-L1 fibroblasts and adipocytes.[9] This indicates that Zn ions mimic insulin functions.

Normal insulin action involves a complicated network of signaling molecules that leads to increased glycogen synthesis, and glucose transport.[10–12] Insulin can act through a variety of signaling pathways to produce its effects. One pathway thought to be involved in glucose and possibly lipid homeostasis leads to activation of the insulin receptor (IR), IR substrates (IRSs) such as IRS-1 and -2, PI3K, and Akt/PKB.[13] Insulin is secreted by the β-cell as a high level spike in response to an immediate glucose load such as a meal[4] and it binds with its specific receptor, IR. This binding becomes a trigger for mediating signaling transduction to the downstream targets of IR, such as IRS and PI3K. Akt/PKB is the downstream target of PI3K signaling and regulates glucose metabolism and transcription.[14] The activated Akt/PKB phosphorylates glycogen synthase kinase-3β (GSK3β), which mediates the glycogen synthesis and regulation of gene expression.[15] Akt/PKB also activates the insulin-dependent glucose transporter 4 (GLUT4) translocation from intracellular compartment vesicle to the plasma membrane (Figure 1).[16]

Previously, our group proposed that Zn complexes in various coordination environments exhibit high in vitro insulin-like and in vivo antidiabetic activities in animals with DM.[17–20] From these studies, we concluded that Zn complexes have higher antidiabetic effects than Zn ions. However, the molecular mechanisms of insulin-like activities of Zn ions or Zn complexes are not fully understood. It has been reported that Zn complexes were also found to act on the IR and PI3K in rat adipocytes, affecting GLUT4 and phosphodiesterase, thereby

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Table 1. Physicochemical Properties of Zn(II) Complexes

Complex (Chemical formula)	Elemental analysis (found/calcd)		Infrared spectra (complex/ligand) ν(C=O)/cm^{-1}	Partition coefficient of Zn complex (log P)
	C/%	H/%		
$[Zn(glc)_2]$ $(C_{12}H_{22}O_{14}Zn\cdot 2.5H_2O)$	28.78/28.78	5.46/5.43	1600/1734	−1.19
$[Zn(trp)_2]$ $(C_{14}H_{10}O_4Zn\cdot 0.5H_2O)$	53.26/53.10	3.63/3.50	1595/1606	0.45
$[Zn(hnk)_2]$ $(C_{20}H_{22}O_4Zn\cdot 0.5H_2O)$	60.16/59.94	6.38/5.78	1591/1608	0.81

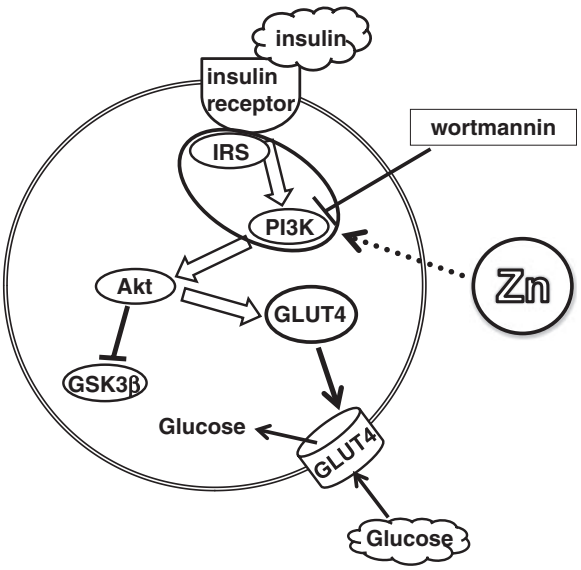
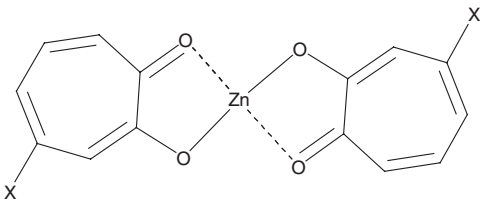


Figure 1. Insulin signaling pathway and the proposed effective point of Zn. Zn complexes activate upstream of PI3K, which follows the activation of downstream cascade. Signaling pathways keys are activation (⇨), inhibition (⊣), stimulation (⇢), and transportation (→).



compounds	X
$[Zn(trp)_2]$	H
$[Zn(hnk)_2]$	*i*-Pr

Figure 2. Estimated structures of Zn complexes.

normalizing the blood glucose levels in experimental animals with DM.[21] Based on the previous reports, it is believed that Zn affects insulin signaling pathways (Figure 1) and may affect GLUT4 translocation.[9,21,22] Therefore, the administration of Zn complexes may be a treatment method of DM. Thus, in this study, we focused on the di(hinokitiolato)zinc complex (hinokitiol: 2-hydroxy-4-isopropylcyclohepta-2,4,6-trienone, $[Zn(hnk)_2]$) showing high insulin-like and antidiabetic effects in in vivo[23] and evaluated the detailed molecular mechanism (the phosphorylation level of Akt/PKB and GSK3β) of $[Zn(hnk)_2]$ using 3T3-L1 adipocytes.

Results

Structural Characteristics of Zn Complexes. Di(tropolonato)zinc(II) (tropolone: 2-hydroxycyclohepta-2,4,6-trienone, $[Zn(trp)_2]$) and $[Zn(hnk)_2]$ were prepared as reported and characterized by several physicochemical methods.[21] The physicochemical parameters of $[Zn(trp)_2]$ and $[Zn(hnk)_2]$ are summarized in Table 1. Structures of these complexes were determined by elemental analyses and Infrared spectra, suggesting that $Zn(O_4)$ coordination mode was present in these complexes at a binding ratio of 1:2 (Zn:ligand). In the Infrared spectra of all gluconic acid, tropolone, and hinokitiol, bands due to O–H and C=O stretching frequencies were found at approximately 3000–3250 and 1600 cm^{-1}, respectively. The O–H stretching frequency bands disappeared and the C=O stretching frequency bands shifted in the Zn(II) complexes (Table 1), and therefore both coordination of the hydroxy group and the carbonyl group were indicated (Figure 2).

The partition coefficients (log P) of all 3 Zn complexes, zinc gluconate ($[Zn(glc)_2]$), $[Zn(trp)_2]$, and $[Zn(hnk)_2]$, were determined in a chloroform/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) system using a conventional method, and the resulting log P were in the following order: $[Zn(hnk)_2]$ (0.81) > $[Zn(trp)_2]$ (0.45) > $[Zn(glc)_2]$ (−1.19) (Table 1).

Stimulation of Akt/PKB Phosphorylation by Zn Complexes in 3T3-L1 Adipocytes. To better understand the signaling pathways affected by Zn complexes, factors of the insulin signaling cascade were examined in non-, dimethyl sulfoxide (DMSO; control)-, insulin- and 25 μM Zn complexes-stimulated 3T3-L1 adipocytes. 3T3-L1 cells are mouse derived fibroblast cells. These fibroblast cells become adipocytes by induction of differentiation. To examine whether Zn complexes induce Akt/PKB phosphorylation, 3T3-L1 adipocytes were treated with 25 μM of each Zn complex or 100 nM insulin for 10 min at 37 °C. Figure 3 shows that 25 μM $[Zn(hnk)_2]$ induced Akt/PKB phosphorylation, with a stronger intensity than that induced by other Zn complexes. After being phosphorylated at serine 473, Akt/PKB becomes active and causes phosphorylation of its downstream substrates, including

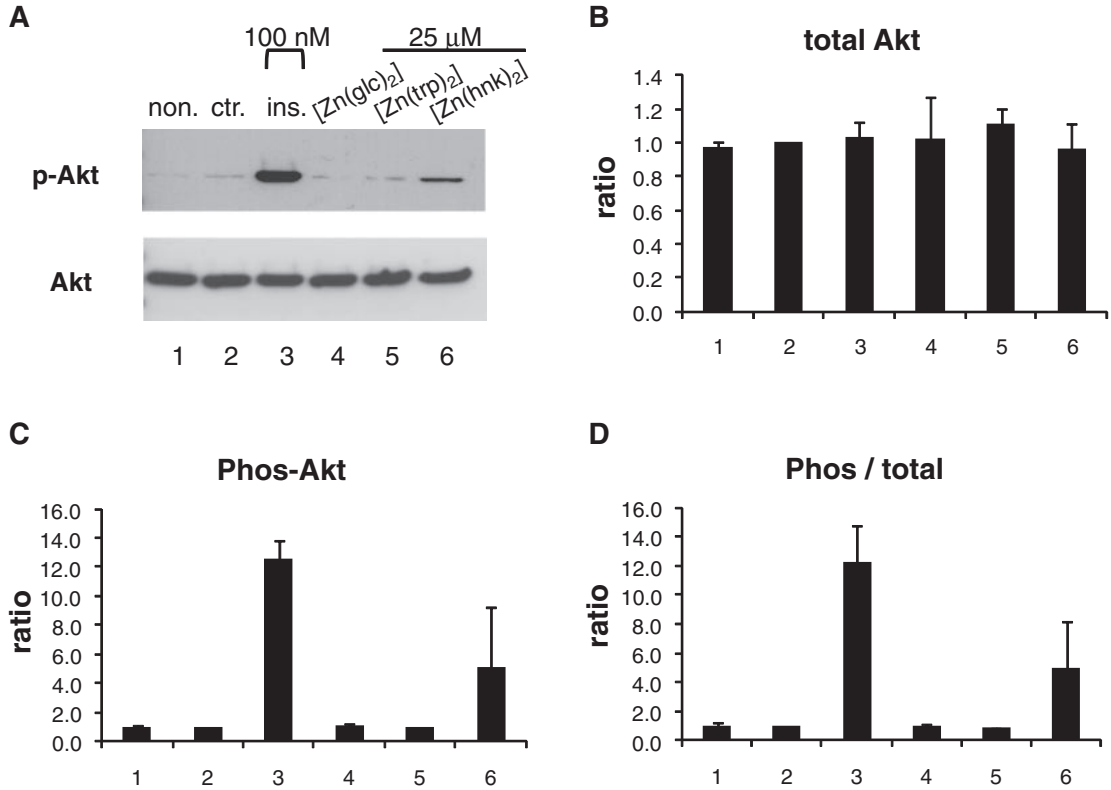


Figure 3. Akt/PKB phosphorylation by Zn complexes. Serum-starved 3T3-L1 adipocytes were treated with 25 μM Zn complexes for 10 min. The cell lysates (10 μg) were separated with 10% SDS-PAGE, and immunoblotted with phospho-Akt (p-Akt) and Akt antibodies. Lanes 1–6 indicate nonstimulation, the control (vehicle), 100 nM insulin, $[Zn(glc)_2]$, $[Zn(trp)_2]$, and $[Zn(hnk)_2]$, respectively. Data are expressed as the means and standard deviations for three separated experiments.

GSK3β, which is a key enzyme involved in glycogen metabolism.[24]

Concentration-Dependent Stimulation of Akt/PKB and GSK3β Phosphorylation by $[Zn(hnk)_2]$ in 3T3-L1 Adipocytes. Because $[Zn(hnk)_2]$-induced Akt/PKB phosphorylation was stronger than that mediated by the other 2 Zn complexes, we focused on $[Zn(hnk)_2]$ complex in our subsequent experiments. To examine whether Akt/PKB or GSK3β phosphorylation is induced in a concentration-dependent manner, the cells were stimulated with different amounts of $[Zn(hnk)_2]$ for 10 min to determine the optimal concentration. In these experiments, we also evaluated the phosphorylation level of GSK3β. Akt/PKB activation increases glycogen synthesis, at least in part, through inhibition (via phosphorylation) of the Akt/PKB substrate, GSK3β. Figure 4a shows that $[Zn(hnk)_2]$ stimulated Akt/PKB phosphorylation in a concentration-dependent manner. With a stimulation period of 10 min, 50 μM $[Zn(hnk)_2]$ induced Akt/PKB phosphorylation strongly. Similarly, $[Zn(hnk)_2]$ stimulated GSK3β in a dose-dependent manner (Figure 4b). These results showed that 50 μM $[Zn(hnk)_2]$ could induce Akt/PKB phosphorylation in 3T3-L1 adipocytes and the extent of phosphorylation due to 50 μM $[Zn(hnk)_2]$ was stronger than that due to 100 nM insulin, suggesting that $[Zn(hnk)_2]$ may stimulate more protein kinases or inhibit protein phosphatases, which dephosphorylate protein kinases.

Inhibition of $[Zn(hnk)_2]$-Induced Akt/PKB Phosphorylation by PI3K Inhibitor, Wortmannin, in 3T3-L1 Adipocytes. To determine whether $[Zn(hnk)_2]$ activates Akt/PKB directly or not, we used a PI3K inhibitor, wortmannin. If $[Zn(hnk)_2]$ activates Akt/PKB directly, it is expected that there would be no measurable difference between the Akt/PKB phosphorylation levels with and without wortmannin. And if $[Zn(hnk)_2]$ does not activate Akt/PKB directly, involving the activation of the upstream proteins in insulin signaling pathway, it is expected that the Akt/PKB phosphorylation level with wortmannin would decrease. 3T3-L1 adipocytes were pretreated in the presence or absence of 100 nM wortmannin for 60 min, followed by treatment with 100 nM insulin or 50 μM $[Zn(hnk)_2]$. Pretreatment with wortmannin suppressed $[Zn(hnk)_2]$-induced Akt/PKB phosphorylation by about 80%. The insulin stimulation sample pretreated with wortmannin was decreased 71% compared to the insulin stimulation sample without wortmannin (Figure 5a, significantly difference: $^{*}p < 0.05$ vs. wortmannin (−)). Furthermore, the pretreatment of wortmannin decreased GSK3β phosphorylation. Both the insulin stimulation and $[Zn(hnk)_2]$ stimulation samples pretreated with wortmannin were less than without wortmannin samples by about 30% (Figure 5b, significant difference: $^{**}p < 0.01$ vs. wortmannin (−)). These results showed that activation of Akt/PKB by $[Zn(hnk)_2]$ was mediated by PI3K because pretreatment of wortmannin in the cells abolished enzyme activation. Consequently, this indicates that $[Zn(hnk)_2]$ activated Akt/PKB indirectly whereby potentially affecting components upstream of PI3K, including IRS-1 or IRβ subunits.

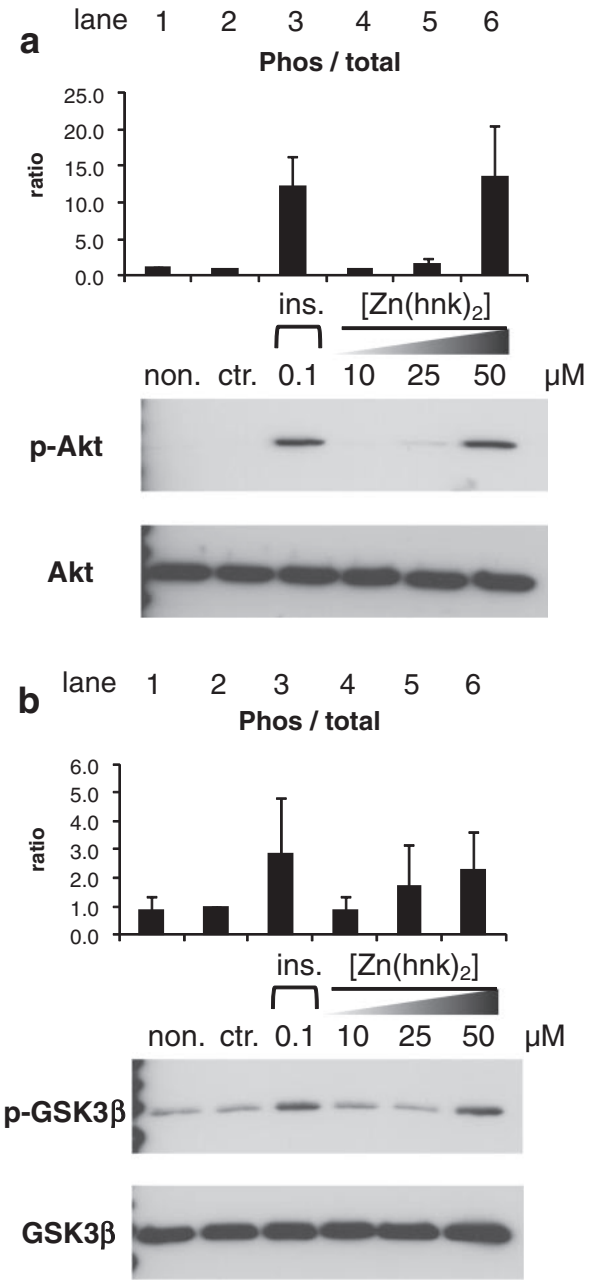


Figure 4. Concentration-dependent Akt/PKB (a) and GSK3β (b) phosphorylation by [Zn(hnk)$_2$] in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes were treated with the different concentrations of [Zn(hnk)$_2$] for 10 min and the cell lysates (10 μg of total protein) were separated with 10% SDS-PAGE, and immunoblotted with phospho-Akt (p-Akt) and Akt antibodies or phospho-GSK3β (p-GSK3β) and GSK3β antibodies. The nonstimulation, control (vehicle), and 100 nM insulin experiments are indicated as non., ctr., and ins. Data are expressed as the means and standard deviations for three separated experiments. Lanes 1–6 indicate nonstimulation, control, 100 nM insulin, 10 μM [Zn(hnk)$_2$], 25 μM [Zn(hnk)$_2$], and 50 μM [Zn(hnk)$_2$], respectively.

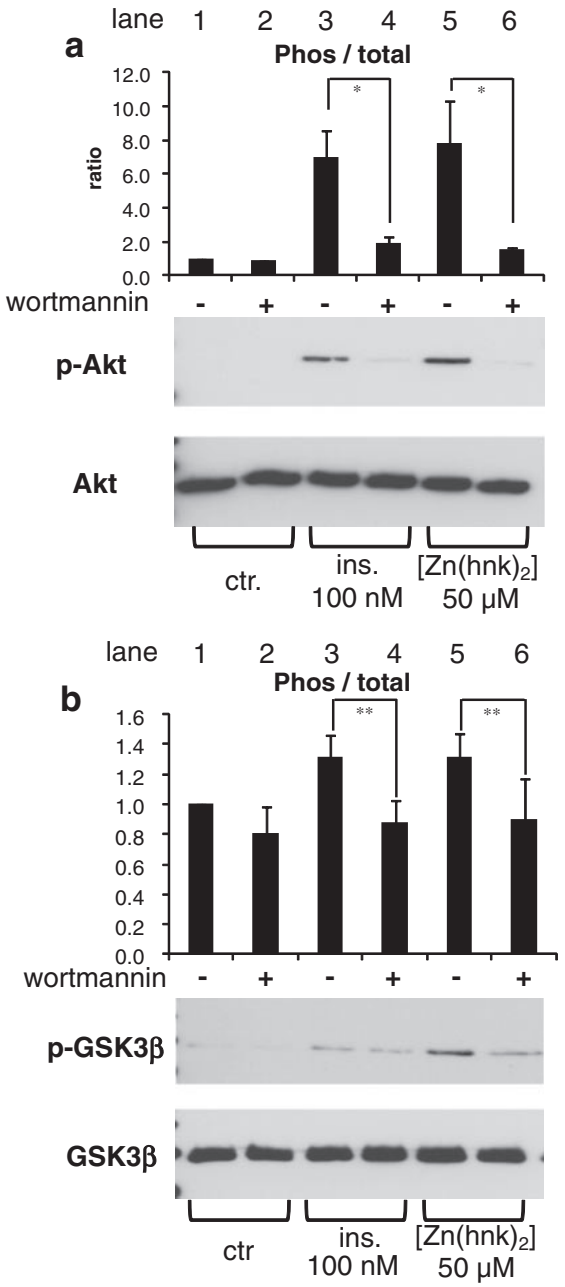


Figure 5. Inhibitory affect of wortmannin on [Zn(hnk)$_2$]-induced both Akt/PKB (a) and GSK3β (b) phosphorylation. The cells were pretreated with (+) or without (−) 100 nM wortmannin for 60 min, then incubated with 100 nM insulin and 50 μM [Zn(hnk)$_2$]. The cell lysates (10 μg of total protein) were separated with 10% SDS-PAGE, and immunoblotted with phospho-Akt (p-Akt) and Akt antibodies or with phospho-GSK3β (p-GSK3β) and GSK3β antibodies. Lane 1: control; lane 2: control with wortmannin; lane 3: 100 nM insulin; lane 4: 100 nM insulin with wortmannin; lane 5: 50 μM [Zn(hnk)$_2$]; lane 6: 50 μM [Zn(hnk)$_2$] with wortmannin. Data are expressed as the means and standard deviations for three separate experiments in Akt/PKB phosphorylation experiments and for five separate experiments in GSK3β phosphorylation experiments. Significant difference: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. wortmannin (−).

Zn Uptake by 3T3-L1 Adipocytes. We showed that 50 μM [Zn(hnk)$_2$] stimulated insulin signaling pathways by inducing Akt/PKB phosphorylation. In this study, we also found that [Zn(hnk)$_2$]-induced Akt/PKB phosphorylation was stronger than the other two Zn complexes (Figure 3). Both [Zn(trp)$_2$] and [Zn(hnk)$_2$] have the troponoid structure, however the Akt/PKB phosphorylation level was different. We hypothesized that this difference comes from the cellular membrane permeability of Zn complexes, and that Zn complexes affect the insulin signaling pathway after penetrating the membrane. Thus, we also examined Zn uptake in the 3T3-L1 adipocytes using atomic absorption spectrometer (AAS), because we did not notice whether Zn gradually affected various proteins in 3T3-L1 adipocytes. The cells were treated with 50 μM [Zn(hnk)$_2$] for 10 min and the complete cell lysates were used to measure Zn uptake. Treatment with 50 μM [Zn(trp)$_2$] and [Zn(hnk)$_2$] resulted in an intracellular Zn concentration of 0.08 and 0.28 μg/10^6 cells, respectively. However, treatment with 1% DMSO resulted in 0.06 μg/10^6 cells. When the unit is converted from μg/10^6 cells to ng/mL cell lysate, concentrations of DMSO, [Zn(trp)$_2$], and [Zn(hnk)$_2$] become 15.2, 22.5, and 77.2 ng mL^{-1} respectively. The value of DMSO (15.2 ng mL^{-1}) is almost equal to an amount reported previously (Figure 6).[22]

In this study, we examined the log *P* of 3 Zn(II) complexes, [Zn(glc)$_2$], [Zn(trp)$_2$], and [Zn(hnk)$_2$]. Their log *P* values were −1.19, 0.45, and 0.81, respectively (Table 1). These values suggest the intracellular transitions of Zn(II) complexes is strongly correlated with the structures and lipophilicity of its ligands. Therefore, the obtained data in this study, log *P* of Zn(II) complexes and Zn uptake (Figure 6), indicate Zn(II) complexes permeate cellular membrane by passive diffusion and affect insulin signaling pathway.

Glucose Uptake for 3T3-L1 Adipocytes. DM is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.[1] Therefore, treatment of DM is needed to improve the blood glucose level. From the above results, [Zn(hnk)$_2$] is expected to have an insulin-like effect. In order to evaluate the antidiabetic effect on improving the blood glucose level, we examined the glucose uptake level by measuring the glucose concentration in phenol-red-free Dulbecco's modified Eagle's medium (DMEM). In the event of increased glucose uptake level obtained from this experiment, the GLUT4 translocation to the membrane will be suspected. From the results of stimulation of Akt/PKB phosphorylation, we concluded that Zn complexes could induce Akt/PKB phosphorylation in a dose-dependent manner (Figure 4). We then evaluated glucose uptake in cells treated with 1% DMSO as the control, 50 μM [Zn(trp)$_2$], or [Zn(hnk)$_2$] for 10 min. 3T3-L1 adipocytes were treated with 1% DMSO, 50 μM [Zn(trp)$_2$], or [Zn(hnk)$_2$] in phenol-red-free DMEM for the indicated time interval and the outer media were collected. Figure 7 shows the glucose uptake level in all the media of cells stimulated with 1% DMSO, 50 μM [Zn(trp)$_2$], or 50 μM [Zn(hnk)$_2$] for 10 min. 50 μM [Zn(hnk)$_2$]-treated adipocytes involved 93.3 mg L^{-1} glucose into the cytoplasm, and the glucose uptake level in [Zn(hnk)$_2$]-treated adipocytes is approximately 3.7-fold higher than that in DMSO.

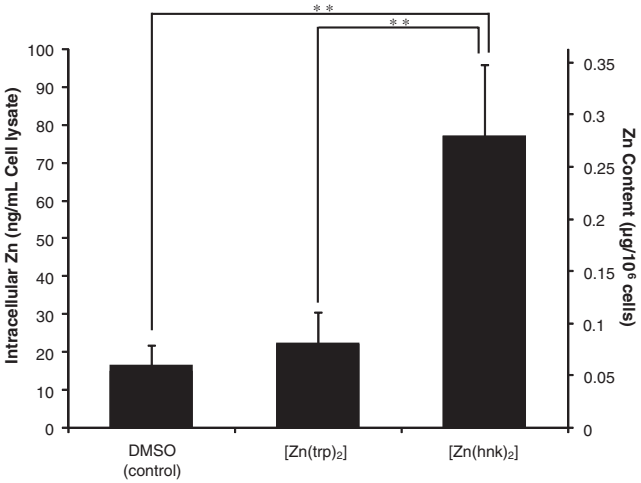


Figure 6. Zn uptake in 3T3-L1 adipocytes treated with [Zn(trp)$_2$] and [Zn(hnk)$_2$]. Serum-starved 3T3-L1 adipocytes were treated with 1% DMSO (control) or 50 μM Zn complexes and incubated at 37 °C for 10 min. The whole cell lysates were used for measuring the Zn uptake. Data are expressed as the means and standard deviations for six to seven experiments. Significantly difference: $^{**}p < 0.01$ vs. control or [Zn(trp)$_2$].

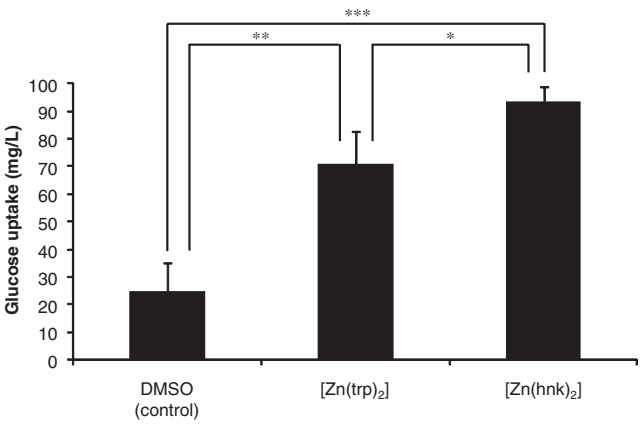


Figure 7. Glucose uptake in 3T3-L1 adipocytes treated with [Zn(trp)$_2$] and [Zn(hnk)$_2$]. 3T3-L1 adipocytes were treated with 50 μM [Zn(trp)$_2$], or [Zn(hnk)$_2$] and incubated at 37 °C for 10 min. The outer media of phenol-red-free DMEM were used for measuring the glucose uptake. Significantly difference: $^{*}p < 0.05$ vs. [Zn(trp)$_2$], $^{**}p < 0.01$ vs. control, $^{***}p < 0.001$ vs. control.

Discussion

As previously reported, Zn and DM are linked at various levels during cellular metabolism.[25] Zn is bound to insulin and was found to have important physiological and pharmacological functions, including insulin-like activities.[26] The diabetic state was also found to accompany Zn deficiency.[27] From the current in vitro and in vivo reports, it can be seen that Zn has insulin-like effects on post-insulin-receptor mechanism and antidiabetic effects.[8,9,28–30] However, these studies used ionic Zn (Zn^{2+}), which has relatively low bioavailability. Thus, other Zn-coordinating compounds were prepared to enhance Zn

bioavailability and its therapeutic potential. We had reported that many types of Zn complexes with different Zn-coordinating environments show antidiabetic effects and lower blood glucose levels significantly in KK-A^y mice with type 2 DM.[17–20] The first orally active antidiabetic Zn complexes were discovered in 2002.[31] Zn-complex formulations make them much easier to permeate cellular membranes because of their enhanced lipophilicity depending on their ligands. They were mainly examined in terms of their insulin-like effects on free fatty acid (FFA)-release inhibition, glucose uptake in vitro, or antidiabetic effects in vivo. Based on these findings, we focused on the [Zn(hnk)$_2$] with a Zn(O$_4$) coordination mode. [Zn(hnk)$_2$] has insulin-like activities in vitro and the complex also promotes normoglycemia, and improves glucose tolerance and insulin resistance in KK-A^y mice with type 2 DM.[23] In this study, we examined the molecular mechanisms whereby the [Zn(hnk)$_2$] complex affects insulin signaling pathways, including Akt/PKB phosphorylation and glucose uptake, because the correlations between DM and Zn are still unclear. Especially how Zn exhibits normoglycemia is unclear. We found that [Zn(hnk)$_2$] has insulin-like effects in vitro in 3T3-L1 adipocytes. Consequently, we confirmed that [Zn(hnk)$_2$] has both antidiabetic and insulin-like effects in KK-A^y mice and 3T3-L1 adipocytes. Among the 3 Zn complexes, [Zn(glc)$_2$], [Zn(trp)$_2$], and [Zn(hnk)$_2$], [Zn(hnk)$_2$] was the best complex of Akt/PKB phosphorylation. We examined the concentration-dependent phosphorylation of Akt/PKB or GSK3β (Figure 4). Next, we evaluated the Akt/PKB and GSK3β phosphorylation in the presence of a PI3K inhibitor, wortmannin, in order to determine whether [Zn(hnk)$_2$] activates Akt/PKB directly or not. As a result, the Zn-induced Akt/PKB phosphorylation was inhibited by wortmannin; meanwhile, GSK3β was suppressed partially by wortmannin (Figure 5). This indicates that the Zn-induced Akt phosphorylation was mediated via PI3K, on the other hand, Zn induced the phosphorylation of GSK3β alternatively; inactivation of GSK3β is due to not only the insulin-dependent PI3K pathway but also other signaling pathways such as mitogen-activated protein kinase (MAPK) cascade and mammalian target of rapamycin (mTOR) pathway mediated by mitogen-activated protein kinase associated protein-1 (MAPKAP-1 (RSK)) and p70 kinase, respectively.[24]

In this study, intracellular Zn concentration incubated with [Zn(hnk)$_2$] for 10 min was found to be approximately 4.7-fold higher than in control cells or 3.5-fold higher than that in cells treated with 50 μM [Zn(trp)$_2$], suggesting that 3T3-L1 adipocytes took up Zn complexes, especially [Zn(hnk)$_2$], into the cytosol and Zn activated Akt/PKB phosphorylation (Figure 6), according well with our previous report showing that Zn uptake in cells treated with Zn complexes was higher than Zn^{2+} uptake.[22] Based on previous reports which indicate Zn(II) complexes have insulin-like activities, it was revealed that Zn(II) complexes with lower stability constants (log β) than 11 exhibited higher insulin-like activities.[32] On the other hand, the Zn(II) complexes, which log β were too low, have the insulin-like activity as small as Zn^{2+}. These data suggest that Zn(II) complexes need moderate stability to have the insulin-like effect and that Zn(II) complexes may affect the insulin-like effect by maintaining the complex structure. Furthermore, glucose uptake was observed when 3T3-L1 adipocytes were stimulated with [Zn(hnk)$_2$] (Figure 7), as was shown previously.[22] This suggested that [Zn(hnk)$_2$] promotes the glucose uptake significantly, that is, [Zn(hnk)$_2$] induces the phosphorylation of Akt/PKB and the promotes the GLUT4 translocations. Therefore, these results prove that [Zn(hnk)$_2$] has the capacity to exert antidiabetic effects. The difference in the glucose uptake level may be the difference in the translocation level in GLUT4, based on the above results (Figures 3–6).

An additional Zn target molecule involved in insulin signaling pathways seems to be GSK3β. GSK3, a serine/threonine kinase that consists of highly homologous α- and β-isoforms,[33] phosphorylates and thereby inactivates glycogen synthase (GS), resulting in reduced glycogenesis. GSK3 activity is associated with decreased insulin sensitivity, and its activity can be acutely inactivated by insulin signaling through IRS-1, PI3K, and ultimately via the action of Akt/PKB to phosphorylate specific serine residues on the enzyme.[34–36] That is, GSK3 decreases insulin sensitivity, and phosphorylated-GSK3 activates the insulin sensitivity. A negative feedback loop in insulin signaling pathways is reported as follows. GSK3 phosphorylates IRS-1, which then inhibits insulin receptor tyrosine kinase activity in vitro and in insulin-resistant rat muscle after insulin stimulation.[35,37] GSK3β level and activity have been shown to be elevated in the muscle of type 2 DM patients, thereby contributing to impaired GS activity and skeletal muscle insulin resistance in type 2 DM patients.[38] It is also reported that inhibition of GSK3 further enhanced basal GS activity and insulin-stimulated glucose transport in insulin-resistant rat skeletal muscle possibly by increasing expression of GLUT4 on the cell surface after insulin stimulation.[35,36,39] Further studies are required to determine the relationship between GSK3 and GLUT4 translocation.

Conclusion

In conclusion, our present results revealed that [Zn(hnk)$_2$] has antidiabetic effects in insulin signaling pathway by inducing phosphorylation of Akt indirectly and inhibiting GSK3β alternatively after intracellular Zn uptake (Figures 3–7). Thus, these events may involve the translocation of GLUT4 to the membrane and glucose uptake.

Experimental

Materials. 3T3-L1 fibroblasts were purchased from DS Pharma Biomedical (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA) and Thermo Scientific (Logan, UT, USA). Newborn calf serum (NCS) and antibiotic/antimycotic compounds were obtained from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin (BSA, protein standard), dexamethasone, and 3-isobutyl-1-methylxanthine were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phospho-Akt (ser473), Akt, phospho-GSK3β (ser9), GSK3β, and horseradish-peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Cell Signaling Technologies (Danvers, MA, USA). Immobilon™ Western Chemiluminescent HRP substrate was from Millipore (Billerica, MA, USA). Wortmannin was obtained from ENZO Life Sciences (Plymouth Meeting, PA, USA). Tropolone was purchased from Tokyo Kasei (Tokyo, Japan). HEPES was obtained from Dojindo (Kumamoto, Japan). [Zn(glc)$_2$], hino-

kitiol, insulin, chloroform, and DMSO for molecular biology were purchased from Wako Pure Chemicals (Osaka, Japan). HNO_3, H_2O_2, $HClO_4$, and standard solutions of Zn for measurements by a graphite furnace AAS were also purchased from Wako Pure Chemicals. [Zn(trp)$_2$] and [Zn(hnk)$_2$] were prepared in our laboratory according to methods reported previously.[23]

Characterization of Zn(II) Complexes and Measurement of the Partition Coefficients (log *P*) of Zn(II) Complexes. The structures of [Zn(trp)$_2$] and [Zn(hnk)$_2$] complexes were estimated by elemental analyses and Infrared spectra. Elemental analyses for carbon and hydrogen were performed by the Analytical Center at Kyoto Pharmaceutical University. The Infrared spectra were measured as KBr disks at 400–4600 cm^{-1} on a Shimadzu FTIR-8400s spectrometer (Shimadzu, Kyoto, Japan).

The log *P* of Zn(II) complexes were determined by the "shake flask method" in a chloroform/HEPES buffer system.[40] After shaking for 1 h at 37 °C, the mixture was allowed to stand for 10 min. The two resulting phases were separated. The concentration of both Zn(II) complexes, [Zn(trp)$_2$] and [Zn(hnk)$_2$], in each phase were monitored at 326 and 250 nm, respectively. We measured the log *P* of [Zn(glc)$_2$] using AAS because [Zn(glc)$_2$] only has poor solubility in chloroform. The partition coefficients were calculated by the equilibrium concentrations of the Zn(II) complexes in chloroform and HEPES buffer.

Cell Culture. 3T3-L1 fibroblasts were cultured in 100-mm Petri dishes under 5% CO_2 at 37 °C in DMEM, supplemented with 10% NCS and antibiotic/antimycotic compounds. Confluent cells were differentiated into adipocytes by adding a mixture of 0.5 mM 3-isobutyl-1-methylxantine, 1 μM dexamethasone, and 10 μg mL^{-1} of insulin in DMEM supplemented with 10% FBS over 72 h. Subsequently, cells were grown in DMEM supplemented with 10% FBS and 10 μg mL^{-1} insulin for 48 h. Thereafter, cells were grown in DMEM supplemented with 10% FBS, and the media were changed every 2 days. Experiments were performed on days 9–16 after inducing differentiation.

Stimulation of 3T3-L1 Adipocytes by Zn Complexes. 3T3-L1 adipocytes were starved in serum-free DMEM for 12 h at 37 °C. These cells were either stimulated with 100 nM insulin or Zn complexes for 10 min at 37 °C. Then, the cells were washed twice in phosphate-buffered saline (PBS), scraped in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM ethylenediamine tetraacetic acid (EDTA), 0.875% Brij-97, 0.125% nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg mL^{-1} leupeptin, 10 mM NaF, and 1 mM $NaVO_3$), and incubated on ice for 20 min. Cell lysates were centrifuged at 15000 rpm for 15 min at 4 °C, and protein concentration was measured using the BCA reagent (Thermo Scientific, UT, USA), including BSA as the standard.

Immunoblotting. Cell lysates (10 μg of total protein) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed using phospho-Akt (ser473), Akt, phospho-GSK3β (ser9), or GSK3β as described previously.[41] The resolved proteins were transferred onto poly(vinylidene difluoride) (PVDF) membranes using iBlot™ gel-transfer systems (Invitrogen, CA, USA), blocked with 5% skim milk in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T), and incubated overnight at 4 °C with primary antibodies in 5% skim-TBS-T. After incubation, the membranes were washed thrice with TBS-T and incubated with HRP-conjugated secondary antibodies. Specific immunoreactions were visualized using an Immobilon™ Western Chemiluminescent HRP substrate and by exposure to Amersham Hyperfilm™ ECL (GE Healthcare UK Ltd., Buckinghamshire, U.K.).

Zn Uptake by 3T3-L1 Adipocytes. 3T3-L1 adipocytes were treated with [Zn(hnk)$_2$] for 10 min at 37 °C, washed in PBS containing 1 mM EDTA, and Zn uptake was determined by using an AAS (AA-6300, Shimadzu, Kyoto, Japan). After washing, the cells were collected and heated at 150 °C with 2 mL of 60% HNO_3, and 2 mL of 60% $HClO_4$, and finally 2 mL of 30% H_2O_2 were added. This procedure was repeated until all organic materials were removed. After cooling, the residues were resuspended in 2 mL of 1% HNO_3 and Zn concentration was measured by AAS, using a calibration curve at a concentration range with linear regression of greater than 0.994 for 5 concentrations. In our experiments, the detection limit of total Zn was 0.1 ng mL^{-1}.

Glucose Uptake by 3T3-L1 Adipocytes. 3T3-L1 adipocytes were stimulated with 100 nM insulin or 50 μM [Zn(hnk)$_2$] in DMEM without phenol red for 10 min at 37 °C. After stimulation, the DMEM was collected and glucose uptake was measured using DRI-CHEM4000sV, an automatic glucose analyzer (Fujifilm, Tokyo, Japan).

Statistical Analysis. Data are expressed as means ± standard deviations. Differences were statistically analyzed by the Student's *t*-test and one-way ANOVA.

Abbreviation

3T3-L1 adipocytes, "3" day "T"ransfer interval, "3" × 10^5 cells per 20 cm^2 dish; AAS, atomic absorption spectrometer; Akt/PKB, Akt/protein kinase B; BSA, bovine serum albumin; DM, diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; FFA, free fatty acid; GLUT4, glucose transporter 4; GS, glycogen synthase; GSK3β, glycogen synthase kinase-3β; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPR, horseradish peroxidase; IR, insulin receptor; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MAPKAP-1, mitogen-activated protein kinase associated protein-1; mTOR, mammalian target of rapamycin; NCS, newborn calf serum; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, poly(vinylidene difluoride); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, tris-buffered saline.

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