Selected Paper

Molecular Mechanism for Antidiabetic Activity of [*meso*-Tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadium(IV) (VO(tpps)) Complex. Studies on Akt Phosphorylation and GLUT4 Translocation in 3T3-L1 Adipocytes

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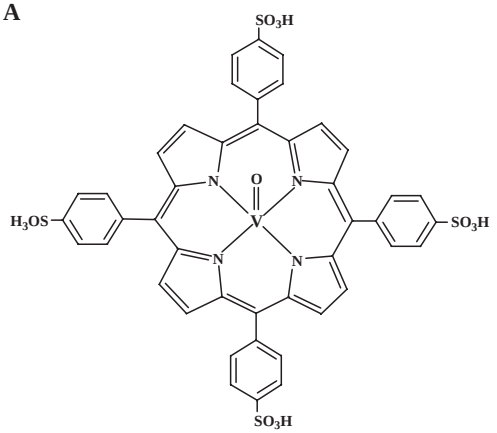
A [*meso*-tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadium(IV) (VO(tpps)), has been found to have an antidiabetic activity and thus to be effective for treating diabetic animal models. High hypoglycemic effects have been observed not only in streptozotocin (STZ)-induced type 1 diabetic mice but also type 2 diabetic KKA^y mice by oral administration of the complex. However, the molecular mechanism for the activities has not yet been examined. The aim of this study was to investigate the molecular mechanism of VO(tpps) in 3T3-L1 adipocytes with respect to insulin signaling pathway. Addition of VO(tpps) was found to phosphorylate the insulin receptor β (IRβ) subunit, insulin receptor substrate 1 (IRS1) and protein kinase B (PKB)/Akt in 3T3-L1 adipocytes, in which all phosphorylations were time dependent. The VO(tpps)-induced Akt phosphorylation was suppressed by a PI3K inhibitor, wortmannin. VO(tpps) also stimulated GLUT4 protein translocation to the plasma membrane. Based on these results, we conclude that VO(tpps) exhibits both insulinomimetic and antidiabetic activities via tyrosine phosphorylations of IRβ and IRS1, which activate the downstream Akt phosphorylation through PI3K, and this signaling cascade finally causes GLUT4 translocation from the cytosol to the plasma membrane.

The antidiabetic or insulinomimetic characters of oxovanadium(IV) have been established in vivo for type 1 as well as type 2 diabetic animal models,[1,2] and together in vitro for rat's adipocytes as well as 3T3-L1 adipocytes.[3,4] Although the in vivo effects of oxovanadium(IV) are not fully understood, this chemical species exhibits antidiabetic activity with respect to hypoglycemic effect, which is also correlated with plasma-lipid lowering effect,[5] enhancing of insulin sensitivity,[6] decreasing of protein tyrosine phosphatase 1 B (PTP1B) activity in rat skeletal muscles,[7] and regulating of glucose transporter 4 (GLUT4) expression.[8] Such in vitro data demonstrate that oxovanadium(IV) affects the insulin signaling pathway with respect to stimulating of insulin receptor autophosphorylation,[1] inhibiting PTP1B,[9] increasing tyrosine phosphorylation of IRS1,[10,11] activating phosphatidylinositol 3-kinase (PI3K),[11] and increasing protein kinase B (PKB)/Akt activity that cause the translocation of the GLUT4 protein from the cytosol to the plasma membrane.[4] In addition, oxovanadium(IV) also inhibited the lipid phosphatase, phosphatase and tensin homologue deleted on chromosome 10 (PTEN),[12] which is the negative regulator for PI3K signaling pathway for regulation of cell growth and survival by dephosphorylating the 3 position of phosphoinositides.[13,14] Based on such multi-functions of oxovanadium(IV), an "ensemble mechanism," in which oxovanadium(IV) acts on at least four sites, involving tyrosine kinase of insulin receptor, PI3K, signal transduction glucose transporter 4 (GLUT4) and phosphodiesterase, emphasizing that the latter two are more important, has been proposed.[15]

Recently, we have developed two oxovanadium(IV) porphyrin complexes, [*meso*-tetrakis(1-methylpyridinium-4-yl)-porphyrinato]oxovanadium(IV) [VO(tmpyp)][16] and [*meso*-tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadium(IV) [VO(tpps)][17,18] (Fig. 1). Both complexes have been proven to be potent antidiabetic complexes in type 1 DM animal models,

A



[*meso*-Tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadium(IV), VO(tpps)
IC_{50} = 0.16 ± 0.01 mM

B **$VOSO_4$**
IC_{50} = 1.00 ± 0.34 mM

Fig. 1. Structure of VO(tpps) used in this study with the IC_{50} value. IC_{50} expresses the concentration of VO(tpps) which inhibits 50% of free fatty acid release from isolated rat adipocytes stimulated by epinephrine (adrenaline).[18]

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in which the antidiabetic activity of VO(tpps) did not require the simultaneous administration of sodium ascorbate compared to that of VO(tmpyp).[17] More recently, VO(tpps) has been shown to be effective in treating type 2 diabetic KKA^{Y} mice through oral administration.[19]

On the basis of these results, we examined the molecular mechanism of VO(tpps) in insulin signaling pathway with respect to Akt phosphorylation and GLUT4 translocation. Akt is a serine/threonine kinase that is a downstream target of PI3K signaling and regulates glucose metabolism, cell proliferation, apoptosis and transcription.[20] Phosphorylated Akt regulates glucose uptake, glycogen synthase kinase 3β (GSK3β) and the forkhead box transcription factor (FOXO) that lead to glycogen synthesis and regulation of gene expression.[21] Akt has been implicated in insulin-stimulated GLUT4 translocation[22] and Akt2, an isoform of Akt, binds to the GLUT4-containing vesicles in response to insulin.[23,24] In addition, mutation of Akt2 in human leads to severe insulin resistance that causes diabetes mellitus.[25]

In this study, we propose that VO(tpps) acts on the insulin signaling cascade through the PI3K/Akt pathway, which in turn translocates the GLUT4 protein to the plasma membrane (Fig. 2). The result is the first finding, in which an oxovanadium(IV) porphyrin complex translocates the GLUT4 protein to the plasma membrane.

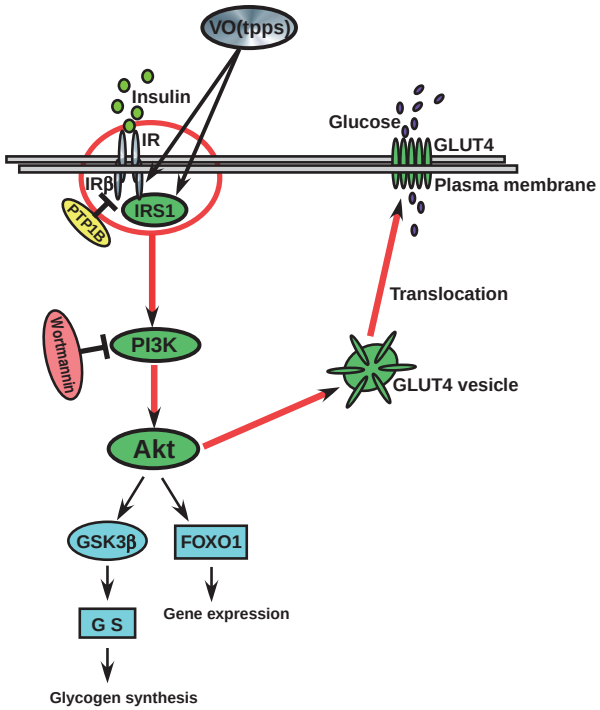

Fig. 2. A proposed mechanism for insulin signaling pathway involving VO(tpps). Addition of VO(tpps) affects the tyrosine phosphorylations of IRβ and IRS1. VO(tpps) might also act on PTP1B, which is the negative regulator for IR, followed by Akt phosphorylation which is mediated by PI3K. Phosphorylated Akt in turn translocates GLUT4 protein from the cytosol to the plasma membrane leading to glucose uptake. In addition, Akt also phosphorylates GSK3β and FOXO1 proteins, which lead to glycogen synthesis and regulation of gene expression, respectively. Thick arrows show the VO(tpps)-stimulated GLUT4 translocation as insulinomimetic activity. Thin arrows show the other target of Akt phosphorylation. The perpendicular symbol ($\perp$) refers to negative regulator or inhibitor.

Experimental

Materials. 3T3-L1 fibroblasts, insulin and fetal bovine serum (FBS) were purchased from ATCC (Rockville, MD, U.S.A.), Wako Pure Chemicals (Tokyo, Japan) and from Equitech Bio (Ingram, TX, U.S.A.), respectively. Bovine serum albumin (BSA, fraction V), dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Antibiotic-antimycotics was purchased from Gibco BRL (San Diego, U.S.A.). Antibodies against phospho-Akt (ser473), Akt, phospho-IRβ (tyr1150/1151), IRβ and HRP-conjugated anti-rabbit IgG were purchased from Cell Signaling Technologies, New England Biolabs (Beverly, MA, U.S.A.). Phospho-IRS1(tyr941) was purchased from Upstate (Charlottesville, VA, U.S.A.). Phospho-GLUT4 antibody was purchased from Calbiochem Merck (Darmstadt, Germany). Alexa Flour 488-labeled secondary antibody was purchased from Molecular Probe (Eugene, OR, U.S.A.). Wortmannin was purchased from BIOMOL Research Laboratories (Plymouth, PA, U.S.A.). H_2tpps, $VOSO_4 \cdot nH_2O$, HNO_3, H_2O_2 and standard solutions of vanadium for the measurement of inductively coupled plasma-mass spectrometry (ICP-MS) were purchased from Wako Pure Chemicals (Osaka, Japan). The $VOSO_4 \cdot nH_2O$ was standardized complexometrically with ethyleneamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) and determined to be the $3.0H_2O$ adduct, and then used in all experiments. VO(tpps) was prepared in our laboratory according to the method reported previously.[17,18] All other reagents were of analytic reagent grade.

Cell Culture. 3T3-L1 fibroblasts were grown in 65 mm diameter dishes under atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. Adding a cocktail of 0.5 mM 3-isobutyl-1-methylxantine, 1 µM dexamethasone and 10 µg mL^{-1} of insulin in DMEM medium for 2 days, followed by growing in DMEM supplemented with 5 µg mL^{-1} insulin for another 2 days induce the differentiation of fibroblast into adipocytes. Then, the cells were grown in DMEM medium, and the medium was changed every second day. The experiments were performed from the 9th to 12th day after differentiation was induced.

Stimulation of 3T3-L1 Adipocytes by VO(tpps). 3T3-L1 adipocytes were starved in DMEM for 15 h at 37 °C, and they were either stimulated with insulin or VO(tpps) and incubated at 37 °C. Then, the cells were washed in PBS and scraped into a cell lysis buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 0.875% Brij 97, 0.125% nonidet P-40 (NP-40), 1 mM PMSF, 5 µg mL^{-1} leupeptin and 10 mM NaF, 1 mM $NaVO_3$, and incubated on ice for 30 min. The cell lysates were centrifuged for 30 min at 4 °C, 12000 rpm. The protein concentration was determined with a Bradford reagent (Bio-Rad, CA, U.S.A.), using BSA as standard.

Immunoblotting Analysis. The cell lysates (15 µg) were separated with 9% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoreses) and immunoblotting analyses with phospho-Akt (ser473), Akt, phospho-IRβ (tyr1150/1151), and phospho-IRS1 (tyr941) were performed as previously described.[4]

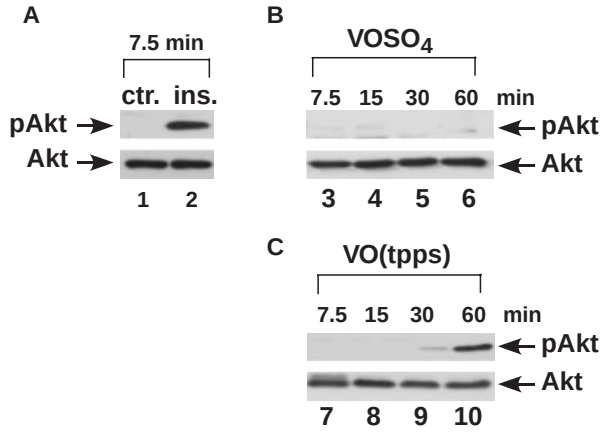

Fig. 3. VO(tpps)-induced Akt phosphorylation (pAkt). The 3T3-L1 adipocytes were stimulated with 100 nM insulin (A), 500 μM $VOSO_4$ (B), and 500 μM VO(tpps) (C) for different stimulating time. The cell lysates (15 μg) were separated with 9% SDS-PAGE and immunoblotted with phospho-Akt (ser473) and Akt antibodies. Left panel indicates the insulin-treatment, lane 1: control (ctr.) and lane 2: insulin (ins.). Right panel shows Akt phosphorylation (pAkt) by $VOSO_4$ (B) and VO(tpps) (C) for different lengths of time. Lanes 3–6 and lanes 7–10 indicate 7.5, 15, 30, and 60 min stimuli.

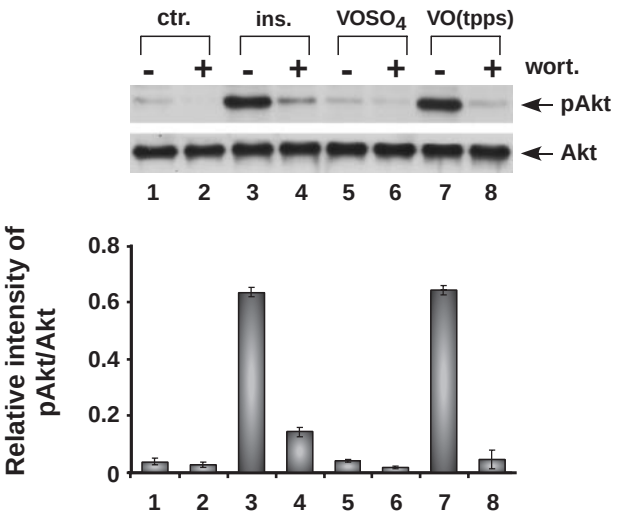

Fig. 4. Suppression of VO(tpps)-induced Akt phosphorylation (pAkt) by wortmannin. The 3T3-L1 adipocytes were pretreated with (+) or without (−) wortmannin (wort.) for 60 min, and then stimulated with 100 nM insulin (ins.), 500 μM $VOSO_4$ or 500 μM VO(tpps) for another 60 min. The cell lysates (15 μg) were separated with 9% SDS-PAGE and immunoblotted with phospho-Akt (ser473) and Akt antibodies. Lane: 1, control (ctr.); lane 2: control with wortmannin; lane 3: 100 nM insulin (ins.); lane 4: 100 nM insulin with wortmannin; lane 5: 500 μM $VOSO_4$; lane 6: 500 μM $VOSO_4$ with wortmannin; lane 7: 500 μM VO(tpps); lane 8: 500 μM VO(tpps) with wortmannin.

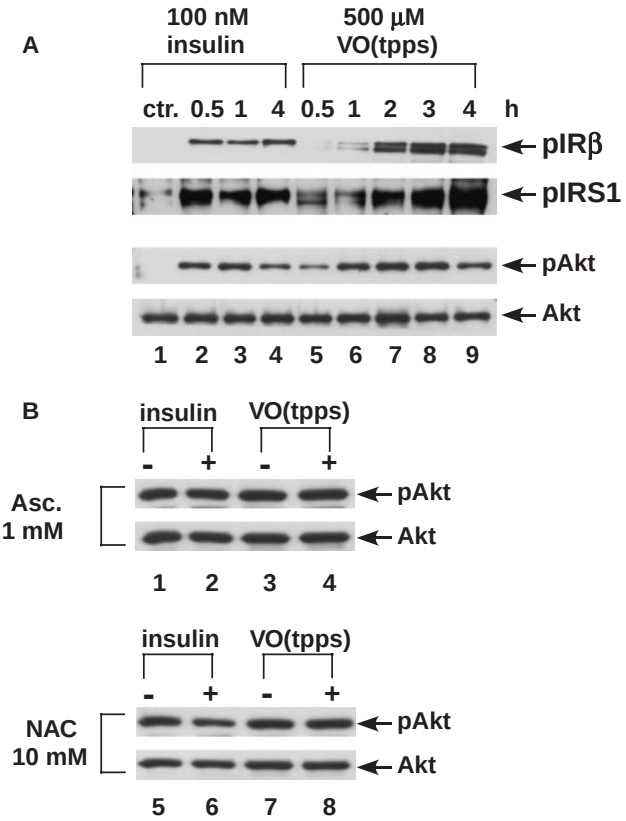

Fig. 5. Time-dependent levels of VO(tpps)-induced IRβ phosphorylation (pIRβ), IRS1 phosphorylation (pIRS1) and Akt phosphorylation (pAkt) (A), and the effect of antioxidants on VO(tpps)-induced Akt phosphorylation (B). (A) Lane 1: control (ctr.); lanes 2–4 indicate 0.5, 1, and 4 h stimuli with 100 nM insulin, respectively. Lanes 5–6 indicate 0.5, 1, 2, 3, and 4 h stimuli with 500 μM VO(tpps). The cell lysate (50 μg) were separated with 9% SDS-PAGE and immunoblotted with phospho-IRβ(tyr1150/1151) and phospho-IRS1(tyr941). (B) The cells were pretreated with (+) or without (−) 1 mM ascorbic acid (Asc.) or 10 mM *N*-acetylcysteine (NAC), and then incubated with 100 nM insulin or 500 μM VO(tpps). Lane 1: 100 nM insulin; lane 2: 100 nM insulin with Asc.; lane 3: 500 μM VO(tpps); lane 4: 500 μM VO(tpps) with Asc.; lane 5: 100 nM insulin; lane 6: 100 nM insulin with NAC; lane 7: 500 μM VO(tpps); lane 8: 500 μM VO(tpps) with NAC; The cell lysate (15 μg) were separated with 9% SDS-PAGE and immunoblotted with phospho-Akt (ser473) and Akt antibodies.

Immunofluorescence Microscopy. 3T3-L1 adipocytes were starved with DMEM for 15 h and stimulated with either insulin, H_2tpps, $VOSO_4$, or VO(tpps) for 60 min and fixed with 4% paraformaldehyde for 15 min. Then, they were washed with ice-cold PBS and permeabilized with PBS containing 0.5% Triton X-100 and 0.2% saponin, followed by blocking in PBS containing 0.2% Triton X-100 and 1% BSA for 30 min, then incubated with anti-GLUT4 antibody at 4 °C for 1 h. After washing extensively, the cells were incubated with Alexa Fluor 488-labeled secondary antibody at 4 °C for 1 h. The cells were visualized by a LSM510 laser confocal microscopy (Carl Zeiss, Tokyo, Japan).

Vanadium Uptake. 3T3-L1 adipocytes were treated with

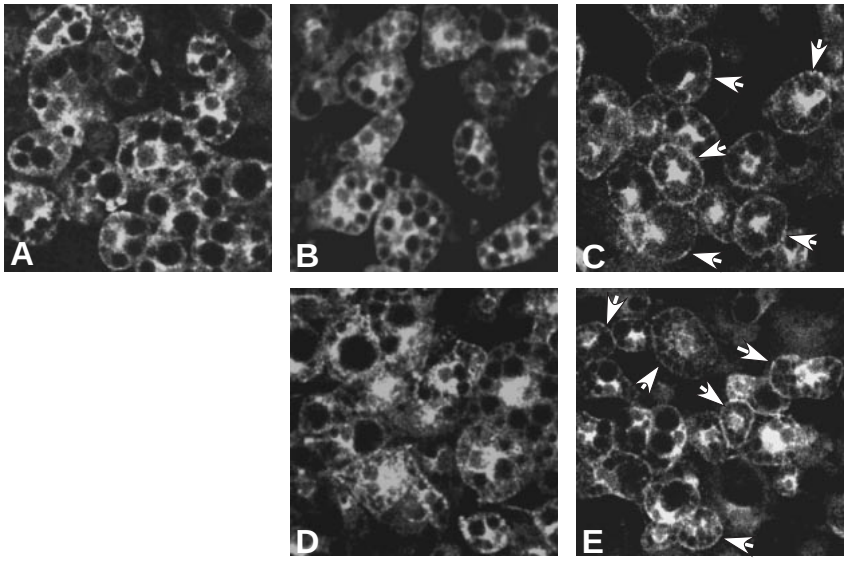

Fig. 6. VO(tpps)-induced GLUT4 translocation from the cytosol to the plasma membrane after 1 h stimulation. The 3T3-L1 adipocytes were treated with 100 nM insulin, 500 μM $VOSO_4$, 500 μM H_4tpps, and 500 μM VO(tpps). Then, the cells were fixed with 4% paraformaldehyde and blocked with 1% BSA, incubated with anti-GLUT4 antibody for 1 h and followed by incubating with Alexa Fluor 488-labeled secondary antibody for 1 h. Panels A–E indicate control, 500 μM $VOSO_4$, 100 nM insulin, 500 μM H_4tpps, and 500 μM VO(tpps), respectively. Arrows indicate the transclocated GLUT4 protein to the plasma membrane.

500 μM VO(tpps) over a time interval at 37 °C. Total vanadium uptake was determined using ICP-MS as previously described.[26] After being treated with VO(tpps), the cells were washed 3 times in PBS containing 1 mM EDTA, followed by centrifugation at 5000 rpm for 5 min and dissolved in 300 μL deionized-distilled water.

Results and Discussion

Stimulation of Akt Phosphorylation by VO(tpps) in 3T3-L1 Adipocytes. Since VO(tpps) exhibited high antidiabetic activity in both types of diabetic animals on oral administration and the ligand alone did not have the activities,[17–19] we examined the effect of VO(tpps) in in vitro experiments by comparing the results to those using $VOSO_4$. To examine whether or not VO(tpps) was able to induce Akt phosphorylation, 3T3-L1 adipocytes were stimulated with different concentrations of VO(tpps), $VOSO_4$, or 100 nM insulin for different stimulating time, up to 60 min. Figure 3 shows that Akt phosphorylation was induced after 60 min stimulation with 500 μM VO(tpps) (Fig. 3C, lane 10). Concentration of VO(tpps) lower than 500 μM did not induce Akt phosphorylation (data not shown). In addition, $VOSO_4$ did not induce Akt phosphorylation under the same conditions (Fig. 3B). The length of time it took VO(tpps) to stimulate Akt phosphorylation is probably due to the fact that VO(tpps) as a macromolecule has a high lipophilic character for penetrating the cell membrane.

Suppression of VO(tpps)-Induced Akt Phosphorylation by Wortmannin in 3T3-L1 Adipocytes. A well-known PI3K inhibitor, wortmannin, at a 100 nM concentration inhibited PI3K activity and GLUT4 translocation by binding irreversibly to class I of PI3K.[27] To determine whether VO(tpps) activates Akt directly or not, we used wortmannin. 3T3-L1 adipocytes were pretreated in the presence (+) or absence (−) of 100 nM wortmannin for 60 min, before treatment with 100 nM insulin, 500 μM $VOSO_4$ or 500 μm VO(tpps) for another 60 min. Pretreatment with wortmannin suppressed the insulin-induced Akt phosphorylation and completely suppressed the VO(tpps)-induced Akt phosphorylation (Fig. 4, lane 8). These results indicate that PI3K mediates the activation of Akt phosphorylation. Consequently, VO(tpps) activates Akt indirectly which might affect the upstream components of PI3K, such as IRS1 and IRβ subunits.

Enhancement of Tyrosine Phosphorylation of IRS1 by VO(tpps) in 3T3-L1 Adipocytes. We next examined the tyrosine phosphorylation of IRS1 and IRβ subunits in 3T3-L1 adipocytes that might be induced by VO(tpps). The cells were stimulated with 500 μM VO(tpps) for different stimulating time. The tyrosine phosphorylations of IRβ subunits and IRS1, and activation of PI3K might play the key roles in mediating the insulin-mimetic effect of VO(tpps) on Akt phosphorylation.[15] The results show that VO(tpps) is able to induce tyrosine phosphorylations of IRS1 and IRβ subunit within a 1 h stimulation (Fig. 5A, first panel, lanes 7–9; second panel, lanes 7–9), and the extent of phosphorylation increased with longer stimulations. Thus, IRβ and IRS1 are the target sites for VO(tpps) in developing insulinomimetic action. Since Akt is a downstream protein of PI3K in the insulin signaling cascade, we also examined the Akt phosphorylation. Figure 5A (third panel) shows that VO(tpps) stimulates Akt phosphorylation in a time-dependent manner, where the phosphorylation is optimum with a stimulation time of 2–3 h. This implies that VO(tpps), which acts as an insulinomimetic agent, targets the peripheral area of the cells by increasing the tyrosine phosphorylations of IRβ and the IRS1.

Next, we examined whether Akt phosphorylation was due to the generation of reactive oxygen species (ROS) by the complex, because ROS is thought to induce Akt phosphorylation.[28] Figure 5B shows that neither ascorbic acid (Asc.) nor *N*-acetylcysteine (NAC) had an effect on Akt phosphorylation, indicat-

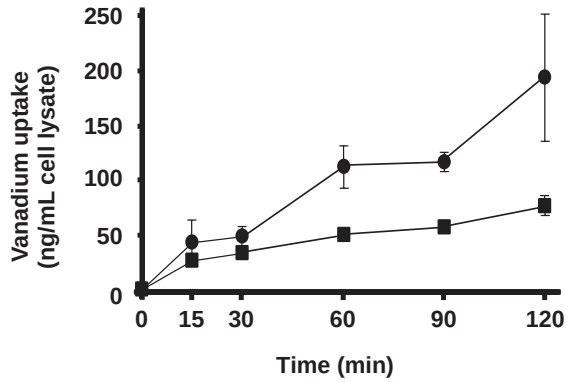

Fig. 7. Time-dependent vanadium uptake in 3T3-L1 adipocytes. The cells were treated with 500 μM $VOSO_4$ and 500 μM VO(tpps) and incubated for different time intervals: 0, 15, 30, 60, 90, and 120 min. The whole cell lysates were used for measuring the vanadium uptake. The symbols indicate as closed square for $VOSO_4$ and closed circle for VO(tpps).

ing that the Akt phosphorylation induced by VO(tpps) is due to the tyrosine phosphorylations of IRβ and IRS.

GLUT4 Protein Translocation to the Plasma Membrane by VO(tpps) in 3T3-L1 Adipocytes. Since the discovery of insulin in the early 1920s, it has taken many decades to understand that insulin stimulates GLUT4 translocation from the cytosol to the plasma membrane. For example, the PI3K/Akt pathway has been intensively studied and shown to be related to GLUT4 translocation.[29,30] Nevertheless, it remains unclear how insulin mobilizes GLUT4-containing vehicles from its intracellular storage to the plasma membrane. Identification and characterization of specific molecules required for both insulin signaling cascade and GLUT4 translocation have been a focus in developing specific therapeutic agents, especially for diabetes mellitus (DM) treatment and prevention.[31] From the obtained results (Figs. 3–5), we concluded that VO(tpps) mimics insulin action through the insulin signaling pathway. Therefore, we examined whether or not VO(tpps) was able to translocate GLUT4 protein from the cytosol to the plasma membrane in 3T3-L1 adipocytes. 3T3-L1 adipocytes were stimulated with 500 μM VO(tpps), 500 μM $VOSO_4$, 500 μM H_2tpps (ligand), or 100 nM insulin for 60 min, and then GLUT4 translocation was detected by using confocal fluorescence microscopy. Figure 6 shows that GLUT4 was detectable on the plasma membrane of the adipocytes which were stimulated with both 100 nM insulin or 500 μM VO(tpps) (Fig. 6, panels C and E). This is the first example to our knowledge showing that VO(tpps) is capable of translocating the GLUT4 protein to the plasma membrane similar to insulin.

Vanadium Uptake in 3T3-L1 Treated with VO(tpps). Next, we examined the vanadium uptake in the cells. The cells were incubated with 500 μM VO(tpps) over different time intervals, and the cell lysates were used to measure the total vanadium uptake. Figure 7 shows that the vanadium uptake increased after treatment for longer than 30 min (Fig. 7, closed circle). In contrast, the cells treated with $VOSO_4$ barely showed any vanadium uptake (Fig. 7, closed square). This result is in agreement with the result of VO(tpps)-induced Akt phosphorylation which was observed after stimulation for 1 h

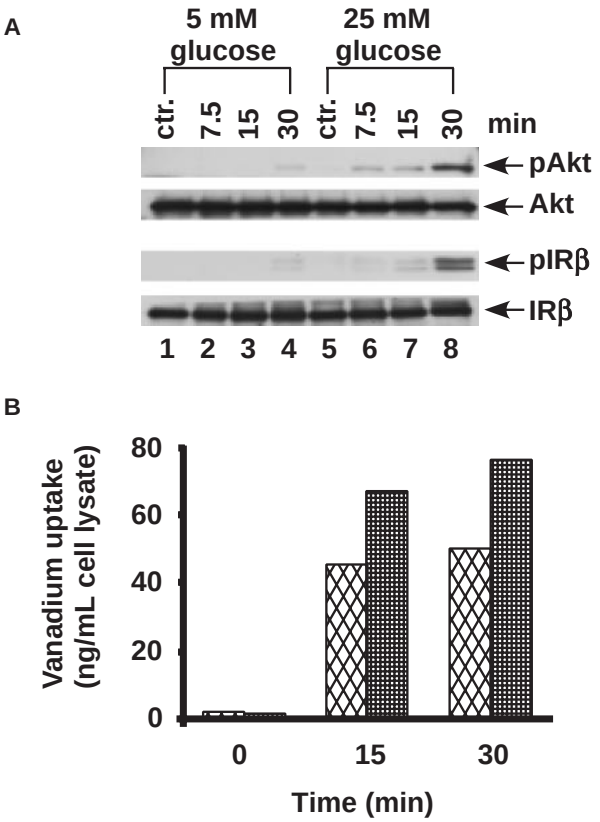

Fig. 8. VO(tpps)-induced Akt phosphorylation (pAkt) in 3T3-L1 adipocytes, in the presence of a large concentration of glucose (A). The 3T3-L1 adipocytes were stimulated with 500 μM VO(tpps) for different amounts of time. The cell lysates (15 μg) were separated with 9% SDS-PAGE, and immunoblotted with phospho-Akt (ser473) and Akt antibodies. Left panels, lane 1: control (ctr.); lanes 2–4 indicate 7.5, 15, and 30 min stimuli with 500 μM VO(tpps), respectively, for the cells grown at 5 mM glucose. Lane 5: control (ctr.); lanes 6–8 indicate 7.5, 15, and 30 min stimuli with 500 μM VO(tpps), respectively, for the cells grown in 25 mM glucose. The vanadium uptake (B) after 0, 15, and 30 min stimuli with 500 μM VO(tpps). Open hatched bar indicates that the cells were grown in 5 mM glucose and the closed hatched bar indicates that the cells were grown in 25 mM glucose.

(Fig. 3C, lane 10).

Enhancement of Akt Phosphorylation by VO(tpps) in 3T3-L1 Adipocytes Grown in the Presence of a Large Amount of Glucose. Finally, we examined the effect of VO(tpps)-induced Akt phosphorylation in the presence of a large concentration of glucose, 25 mM, to mimic insulin resistance. Recently, we have demonstrated that VO(tpps) has a high hypoglycemic effect in treating both types of diabetic animals. Interestingly, as shown in Fig. 8, VO(tpps) enhanced the phosphorylation of Akt better in 25 mM glucose-treated cells than that in 5 mM glucose-treated cells (Fig. 8A). Under the same conditions, 25 mM glucose-treated cells had a slightly greater vanadium uptake than that of 5 mM glucose-treated cells (Fig. 8B).

On the basis of the results, we concluded that VO(tpps) exhibits both insulinomimetic and antidiabetic activities by tyro-

sine phosphorylations of IRβ and IRS1, which phosphorylate the Akt through PI3K signaling pathway, and this signaling cascade finally causes GLUT4 translocation from the cytosol to the plasma membrane. In addition, VO(tpps) was more effective in the cells grown in a high glucose concentration, which mimics insulin resistance that is found in type 2 DM.

Abbreviations

VO(tpps), [*meso*-tetrakis(4-sulfonatophenyl)phorphyrinato]-oxovanadium(IV); GLUT4, glucose transporter 4; IRβ, insulin receptor beta subunit; IRS1, insulin receptor substrate 1; GSK3β, glycogen synthase kinase 3β; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

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