We previously isolated dephostatin from Streptomyces as a novel inhibitor of CD45-associated protein-tyrosine phosphatase. We prepared Et-3,4-dephostatin as a stable analogue and found it to inhibit PTP-1B and SHP-1 protein-tyrosine phosphatases selectively but not to inhibit CD45 and leukocyte common antigen-related phosphatase ones effectively. Et-3,4-dephostatin increased the tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 with or without insulin in differentiated 3T3-L1 mouse adipocytes. The increase of tyrosine phosphorylation by Et-3,4-dephostatin was more prominent in 6-h than in 30-min incubation. It also increased phosphorylation and activation of Akt with or without insulin. Et-3,4-dephostatin also enhanced translocation of glucose transporter 4 from the cytoplasm to the membrane and 2-deoxy-glucose transport. Et-3,4-dephostatin-induced glucose uptake was inhibited by SB203580, a p38 inhibitor, but not by PD98059, a MEK inhibitor, or by cycloheximide as insulin-induced uptake. Interestingly, although LY294002, a phosphatidylinositol 3-kinase inhibitor, inhibited the insulin-induced glucose uptake completely, it only partially inhibited the Et-3,4-dephostatin-induced uptake. It also blocked insulin-induced glucose transporter 4 translocation but not the Et-3,4-dephostatin-induced one. The increase in c-Cbl tyrosine phosphorylation caused by Et-3,4-dephostatin was stronger than that in insulin receptor phosphorylation. These observations indicate that a phosphatidylinositol 3-kinase-independent pathway involving c-Cbl is more important in Et-3,4-dephostatin-induced glucose uptake than in insulin-induced uptake. Et-3,4-dephostatin showed an in vivo anti diabetic effect in terms of reducing the high blood glucose level in KK-Ay mice after oral administration. Thus, Et-3,4-dephostatin potentiated insulin-related signal transductions in cultured mouse adipocytes and showed an anti diabetic effect in mice.

Type 2 diabetes mellitus is mainly characterized by impaired signal transduction downstream of the insulin receptor in peripheral tissues, such as skeletal muscles and adipocytes (1–4). Insulin secretion from the pancreatic β-cells is also affected in many cases of type 2 diabetes mellitus (5, 6), since the secondary effect of impaired signal transduction may affect insulin secretion (it was reported that long-term exposure to a high glucose concentration induced apoptosis in pancreatic β-cells (7)). A functional insulin receptor was also shown to be essential for the secretion of insulin by cultured mouse β-cells (8). Thiouazolidine derivatives such as troglitazone are being or have been widely used for the treatment of type 2 diabetes mellitus (9). Troglitazone is a ligand of peroxisome proliferator-activated receptor γ that transcriptionally regulates a number of adipose tissue-specific genes by binding to peroxisome proliferator-activated receptor response elements of these genes as a heterodimer with retinoid X receptor. These heterodimers potentiate adipose tissue differentiation in vivo (10), and their side effects include obesity. Several drugs such as sulfonylureas enhance insulin secretion. Sulfonylureas bind to receptors on pancreatic β-cells to induce Ca2+ influx, thereby increasing insulin secretion (11). Compared with the insulin therapy for type 1 diabetes mellitus, chemotherapy for type 2 has been poorly developed, and less toxic chemotherapy based on the insulin receptor-associated signal transduction should be developed.

The insulin receptor is a heterotetramer consisting of two α-subunits that are entirely extracellular and two β-subunits that span the plasma membrane and contain intrinsic tyrosine kinase activity (12, 13). Binding of insulin to its receptor results in activation of the receptor tyrosine kinase and receptor autophosphorylation, followed by tyrosine phosphorylation of IRS-1, -2, and -3, which then bind to the p85 regulatory subunit of phosphatidylinositol (PI)3 kinase. The PI3 kinase then catalyzes the production of phosphatidylinositol 3,4-bisphosphate, which can bind to the PH domain of Akt (14). These Akts are phosphorylated to become activated by 3-phosphoinositide-dependent protein kinase (PDK)1 and 2. Compared with the insulin receptor-activated response elements of these genes as a heterodimer with retinoid X receptor. These heterodimers potentiate adipose tissue differentiation in vivo (10), and their side effects include obesity. Several drugs such as sulfonylureas enhance insulin secretion. Sulfonylureas bind to receptors on pancreatic β-cells to induce Ca2+ influx, thereby increasing insulin secretion (11). Compared with the insulin therapy for type 1 diabetes mellitus, chemotherapy for type 2 has been poorly developed, and less toxic chemotherapy based on the insulin receptor-associated signal transduction should be developed.

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Potentiation of Insulin-related Signal Transduction

Kandror (18) reported that the phosphorylated Akt2 directly binds to GLUT4-containing vesicles to phosphorylate GLUT4 to trigger translocation of GLUT4 from the cytoplasm to the membrane (19), resulting in an increase in cellular glucose uptake (20). On the other hand, very recently, Pessin and co-workers (21) showed the existence of a PI 3-kinase-independent c-Cbl pathway in insulin-induced signal transduction. The c-Cbl protein is a proto-oncogene product that can be tyrosine-phosphorylated. The Cbl-associated protein forms complex with c-Cbl. This complex mediates GLUT4 translocation through the PI 3-kinase-independent pathway in 3T3-L1 adipocytes (21).

Several protein-tyrosine phosphatases (PTPases) have been identified in major insulin-sensitive tissues such as skeletal muscle, liver, and adipose tissue. These include transmembrane PTPases such as CD45 and leukocyte common antigen-related phosphatase (LAR)/VPTP-α and nontransmembrane PTPases such as SHPTP-1, SHPTP-2, PTP-1B, and PTP-1C (22). Various PTPases are considered to be involved in the etiology of diabetes mellitus. Especially, PTP-1B, a cytoplasmic PTPase, is known to be a negative regulator of insulin receptor-associated signal transduction (23). PTP1B-deficient mice showed increased insulin sensitivity and resistance to obesity (24). Overexpression of PTP-1B in Rat1 fibroblasts is known to reduce ligand-stimulated autophosphorylation of the insulin receptor (23). Therefore, inhibitors of PTP-1B may enhance the insulin sensitivity and glucose uptake.

We previously isolated dephostatin from Streptomyces as a novel inhibitor of T-cell receptor-associated protein-tyrosine phosphatase CD45 (26). Since dephostatin was unstable in cell culture media, later we synthesized Me-3,4-dephostatin as a stable analogue (27). Me-3,4-dephostatin was shown to enhance nerve growth factor- or epidermal growth factor-induced tyrosine phosphorylation of cellular proteins, activation of Akt, enhancement of insulin sensitivity and glucose uptake. Thus, we previously isolated dephostatin from Streptomyces as a novel inhibitor of T-cell receptor-associated protein-tyrosine phosphatase CD45 (26). Since dephostatin was unstable in cell culture media, later we synthesized Me-3,4-dephostatin as a stable analogue (27). Me-3,4-dephostatin was shown to enhance nerve growth factor- or epidermal growth factor-induced tyrosine phosphorylation of cellular proteins, activation of Akt, enhancement of insulin sensitivity and glucose uptake. Me-3,4-dephostatin was shown to enhance nerve growth factor- or epidermal growth factor-induced tyrosine phosphorylation of cellular proteins, activation of Akt, enhancement of insulin sensitivity and glucose uptake.

EXPERIMENTAL PROCEDURES

Materials—Dephostatin analogues were synthesized as described previously (29). Insulin and p-nitrophenyl phosphate were obtained from Sigma. Human recombinant PTP-1B, SHPTP-1, and anti-phosphotyrosine antibody (4G10) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). CD45 and LAR were purchased from Biomol (Plymouth Meeting, PA). Anti-insulin receptor β subunit antibody, anti-Akt antibody, anti-GLUT1 antibody, anti-GLUT4 antibody, and horse-radish peroxidase-conjugated anti-Goat IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphorylated Akt antibody was obtained from New England Biolabs (Mississauga, Canada). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-rabbit IgG were obtained from Amersham Pharmacia Biotech. Western Blot Chemiluminescence Reagent and Tyramide Signal Amplification (TSA™ Direct) were obtained from PerkinElmer Life Sciences. 2-[3H]Deoxy-g-glucose was obtained from American Radiolabeled Chemicals (St. Louis, MO). LY294002 was obtained from Cayman Chemical (Ann Arbor, MI). Protein-G-agarose was obtained from Oncogene Research Products (Cambridge, MA). A Triglyceride G-Test Kit was obtained from Wako (Osaka, Japan).

Cell Culture and Differentiation—3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum at 37°C (28). For differentiation, cells (1 × 10⁶) were cultured in a 60-mm dish for 3 days to postconfluence in medium containing 10% fetal bovine serum, 500 μM isobutylmethylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin. Then the cells were grown in medium containing only 10% fetal bovine serum and insulin for another 2 days. After that, the medium was changed, and the cells were cultured in 10% fetal bovine serum medium for another 5–7 days for full differentiation (30).

Enzyme Assay—PTP-1B, SHPTP-1, CD45, and LAR were assayed in a colorimetric assay kit. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes. After having been blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline-Tween buffer containing 20 mM Tris (pH 7.6), 0.14 M NaCl, and 0.1% (w/v) Tween 20, the membranes were treated with antibodies, and then the proteins were visualized by use of an ECL Western blotting detection system.

Plasma Membrane Sheet Assay—Plasma membrane sheets were prepared from differentiated 3T3-L1 adipocytes as described by Robinson et al. (31). Briefly, the cells cultured on a microcover glass were washed once with ice-cold PBS and incubated with 500 μl of 0.5 mg/ml poly-l-lysine for 30 s. The cells were then washed in 500 μl of hypotonic buffer (293 mM, 5 mM HEPES, pH 7.5, 5 mM EDTA) with three successive rinses. The swollen cells were sonicated for 5 s in a sonication buffer (70 mM KCl, 30 mM HEPES, pH 7.5, 5 mM MgCl₂, 3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The bound plasma membrane sheets were washed three times with the sonication buffer and used for immunofluorescence studies.

2-Deoxyglucose Uptake in 3T3-L1 Adipocytes—Differentiated 3T3-L1 adipocytes were grown in 12-well plates. After preincubation with the desired test chemical for 1 h, 10 nM insulin was added to the medium. After the incubation, the cells were incubated in assay buffer consisting of 140 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.9 mM CaCl₂, 0.5 mM MgCl₂, and 1 mg/ml bovine serum albumin, pH 7.0, at 37°C for 10 min, and then the cells were incubated with 0.01 μg/ml of [3H]Deoxy-g-glucose for 10 min. The uptake was terminated by adding 500 μl of ice-cold PBS containing 0.1 mM phloretin. The cells were washed twice with ice-cold PBS and then solubilized in 0.5 n NaOH. Thereafter, the radioactivity was measured by a scintillation counter. Non-specific glucose uptake was measured by treating cells with 20 μg/ml cytochalasin B, and this value was subtracted from all of the data.

Immunostaining of GLUT4—Immunostaining of GLUT4 was carried out as previously described (32). Differentiated 3T3-L1 adipocytes were cultured on microcover glasses in 12-well plates. After having been treated with 10 μg/ml Et-3,4-dephostatin for 6 h, the adipocytes were fixed in 500 μl of 2% formaldehyde in PBS for 5 min on ice and 5 min at room temperature. Then they were washed with 500 μl of 100 μg/ml glycine in PBS for 15 min and blocked with 500 μl of 5% calf serum in PBS for 15 min at room temperature. The cells were incubated with 2 μl/ml polyclonal anti-GLUT4 antibody in PBS overnight at 4°C. After that, the cells were incubated with horseradish peroxidase-conjugated anti-goat IgG (diluted 1:100 with PBS) at room temperature for 60 min and then washed with TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) three times. The cells were then incubated with fluorophore tyramide (diluted 1:50 with the amplification diluent; PerkinElmer Life Sciences) for 7 min at room temperature in the dark, washed three times, 10 min...
Potentiation of Insulin-related Signal Transduction

Inhibition of PTPases by Et-3,4-dephostatin—PTPases such as CD45, LAR/RPTP-α, SHPTP-1, SHPTP-2, PTP-1B, and PTP-1C are known to be involved in the regulation of the insulin receptor (22).

First, we examined the inhibition of these PTPases by newly prepared Et-3,4-dephostatin (Fig. 1). Et-3,4-dephostatin strongly inhibited PTP-1B and SHPTP-1, the IC₅₀ values being 0.58 and 0.96 µg/ml, respectively. It weakly inhibited CD45 but had no effect on LAR. Therefore, Et-3,4-dephostatin showed selective inhibitory activity on PTP-1B and SHPTP-1. 4-O-Me-Et-3,4-dephostatin (Fig. 1) was synthesized as a negative control. As expected, it did not inhibit any of the PTPases effectively, as shown in Table I.

Effect of Et-3,4-dephostatin on Intracellular Tyrosine Phosphorylation in 3T3-L1 Adipocytes—Mouse 3T3-L1 fibroblasts were differentiated into adipocytes for 8–12 days. Et-3,4-dephostatin was added to the differentiated 3T3-L1 adipocytes (day 10) with or without suboptimal 10 nM insulin; the cells were incubated for 30 min, 2 h, or 6 h; and the tyrosine phosphorylation was then examined by immunoprecipitation with anti-insulin receptor and anti-IRS-1 antibodies. Insulin at 10 nM induced tyrosine phosphorylation of the 95-kDa insulin receptor and IRS-1 in a 30-min incubation. In a 6-h incubation, insulin and Et-3,4-dephostatin also increased the phosphorylation of other proteins at 135, 115, 85, and 60 kDa, which were identified to be phospholipase C-γ, c-Cbl, PI 3-kinase regulatory subunit, and IRS-3, respectively, by the mobility of each authentic protein in a Western blotting analysis (data not shown).

Effect of Et-3,4-dephostatin on Akt Activation—PKB/Akt is a downstream signal transducer of the IR-PI3K pathway that up-regulates insulin signaling. So we examined whether Et-3,4-dephostatin could induce Akt activation, which can be monitored by phosphorylation. We employed a 6-h incubation, since the effect of Et-3,4-dephostatin on tyrosine phosphorylation was more prominent in 6-h incubation. As a result, Et-3,4-dephostatin induced Akt phosphorylation and enhanced insulin-induced activation synergistically, as shown in Fig. 3, whereas it did not alter the amount of Akt. As expected, either insulin-induced or Et-3,4-dephostatin-induced Akt activation was inhibited by LY294002, a PI 3-kinase inhibitor.

Effect of Et-3,4-dephostatin on GLUT4 Translocation—Insulin induces GLUT4 translocation from intracellular vesicles to the plasma membrane (20), so we studied the effect of Et-3,4-
dephostatin on GLUT4 translocation by employing the plasma membrane sheet assay. In this assay, the upper membrane is removed after treatment of the cells, and translocation to the membrane can be detected by the antibody to the intracellular portion of GLUT4 (31). As shown in Fig. 4A, Et-3,4-dephostatin or insulin induced translocation of GLUT4 to the membrane in 3T3-L1 adipocytes in 6 h. GLUT1 was located at the membrane without stimulation, and the location was not altered by Et-3,4-dephostatin or insulin (Fig. 4B). As shown in Fig. 4C, the cellular amount of GLUT4 or GLUT1 was not changed by insulin or Et-3,4-dephostatin. Thus, Et-3,4-dephostatin specifically induced translocation of GLUT4, like insulin.

**Effect of Et-3,4-dephostatin on 2-[³H]Deoxy-d-glucose Uptake**—Glucose uptake is a major phenotypic change induced by

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**Fig. 4.** Effect of Et-3,4-dephostatin on the localization of GLUT4 and GLUT1. 3T3-L1 adipocytes were treated with 10 nM insulin, 10 µg/ml Et-3,4-dephostatin, insulin and Et-3,4-dephostatin, or 10 µg/ml 4-O-Me-Et-3,4-dephostatin for 6 h. Plasma membrane sheets were then prepared and fixed with 2% formaldehyde. The fixed membrane sheets were stained with polyclonal anti-GLUT4 (A) or anti-GLUT1 (B) antibody. The protein levels of GLUT4 and GLUT1 were assayed by Western blotting (C).
insulin in the target tissues. As shown in Fig. 5A, insulin at 10 nM induced glucose uptake most prominently at 30 min. On the other hand, Et-3,4-dephostatin at 10 μg/ml alone induced glucose uptake time-dependently up to 6 h. When added together, Et-3,4-dephostatin increased the uptake additively and time-dependently up to 6 h. Fig. 5B shows the dose effect of Et-3,4-dephostatin on glucose uptake. After incubation, the cells were incubated with 2-[3H]deoxy-D-glucose for 10 min, and the reaction was then terminated. After having been washed twice, the cells were solubilized and neutralized, and the radioactivity was measured by a scintillation counter. Values are means ± S.D. of triplicate determinations.

Inhibition of Et-3,4-dephostatin-induced 2-[3H]deoxy-D-glucose Uptake by Signal Transduction Inhibitors—Next we studied which signaling pathways are involved in Et-3,4-dephostatin-induced glucose uptake in 3T3-L1 adipocytes by using enzyme inhibitors of small molecular weight. 3T3-L1 adipocytes were pretreated with various inhibitors for 1 h and then stimulated with 10 nM insulin and/or 10 μg/ml Et-3,4-dephostatin for 6 h. As shown in Fig. 6A, insulin- or Et-3,4-dephostatin-stimulated glucose uptake was not affected by 50 μM PD98059, a MEK inhibitor, or 30 μg/ml cycloheximide. SB203580, a p38 inhibitor, at 10 nM inhibited both insulin and Et-3,4-dephostatin-stimulated glucose uptake significantly. On the other hand, although 100 μM LY294002, a PI 3-kinase inhibitor, completely inhibited the insulin-stimulated glucose uptake, it only partially inhibited the Et-3,4-dephostatin-stimulated glucose uptake. Fig. 6B shows the effect of LY294002 on insulin- and Et-3,4-dephostatin-induced glucose uptake in 30 min. Unexpectedly, LY294002 inhibited both insulin-induced and the inhibitor-induced glucose uptake completely. Therefore, in long term incubation, Et-3,4-dephostatin may employ different signals from those of insulin for activation of glucose transport, which are independent of PI 3-kinase.

Effect of LY294002 on Et-3,4-dephostatin-induced GLUT4 Translocation—GLUT4 can be translocated without activation of PI 3-kinase (21). Therefore, first we examined the effect of LY294002 on Et-3,4-dephostatin-induced GLUT4 translocation in 3T3-L1 adipocytes, employing the anti-GLUT4 antibody that recognizes the extracellular domain. LY294002 inhibited the insulin-induced translocation of GLUT4 but not the Et-3,4-dephostatin-induced translocation in a 6-h incubation, as shown in Fig. 7. On the other hand, LY294002 inhibited Et-3,4-dephostatin-induced Akt phosphorylation effectively (Fig. 3). Therefore, Et-3,4-dephostatin is likely to activate a PI 3-kinase-independent pathway to mediate GLUT4 translocation and glucose uptake.

Effect of Et-3,4-dephostatin on c-Cbl Phosphorylation—Cellular Cbl was reported to be an important mediator of the PI 3-kinase-independent signaling pathway induced by insulin (21). Therefore, we studied the effect of Et-3,4-dephostatin on c-Cbl phosphorylation in 3T3-L1 adipocytes by immunoprecipitation. As shown in Fig. 8, insulin induced tyrosine phosphorylation of c-Cbl at 10 min, but the phosphorylation disappeared at 6 h. When the cells were treated with Et-3,4-dephostatin with or without insulin, the phosphorylation was clearly observed after 6 h. This phosphorylation of c-Cbl was not inhibited by LY294002. These results were parallel to those of 2-deoxyglucose uptake in Fig. 6. Thus, Et-3,4-dephostatin is likely to inhibit tyrosine dephosphorylation of c-Cbl, activating PI 3-kinase-independent signaling for GLUT4 translocation.

Effect of Et-3,4-dephostatin on Blood Glucose Level in Vivo—Due to the malfunction of their melanocortin receptor, KK-A<sup>v</sup> mice have yellow hair and show obesity and a high blood glucose level of 400–500 mg/dl (33). When Et-3,4-dephostatin was given to KK-A<sup>v</sup> mice at 500 mg/kg orally with the diet, the glucose level significantly decreased by about 50%, as shown in Fig. 9. In this model, troglitazone at 500 mg/kg also lowered the glucose level by about 50% (data not shown). Thus, Et-3,4-dephostatin decreased the blood glucose level in vivo.

**DISCUSSION**

Few PTPase inhibitors have been used as biochemical tools except sodium vanadate. We previously developed Me-3,4-dephostatin, a stable analogue of dephostatin (27) and showed it to enhance the effect of nerve growth factor and epidermal growth factor on differentiation of rat pheochromocytoma PC12 cells (28). In this model, we demonstrate that an additional stable analogue, Et-3,4-dephostatin, potentiated or mimicked the effect of insulin in differentiated 3T3-L1 adipocytes and showed an antidiabetic effect in vivo.

First, we found that Et-3,4-dephostatin selectively inhibits PTP-1B and SHPTP-1, both of which are known to be involved
in the regulation of the insulin receptor (22, 23), as shown in Table I. It only weakly inhibited CD45. The IC₅₀ value for CD45 is larger than that in our previous report (27). This difference occurred because we changed the enzyme source to a commercially available preparation from the crude preparation of Jurkat cell membranes. Et-3,4-dephostatin may inhibit other untested PTPases; therefore, involvement of PTPases other than PTP-1B and SHPTP-1 cannot be excluded in its cellular effects.

Et-3,4-dephostatin increased the phosphorylation of Akt, GLUT4 translocation, and hexose transport in 3T3-L1 adipocytes. 4-O-Me-Et-3,4-dephostatin did not induce these phenotypic changes, indicating that these effects are due to the inhibition of PTPases. Since protein synthesis was blocked by

![FIG. 6. Effect of signal transduction inhibitors on Et-3,4-dephostatin-induced glucose uptake. A, the cells were pretreated with various inhibitors for 1 h and then stimulated with 10 nM insulin and/or 10 μg/ml Et-3,4-dephostatin for 6 h. After incubation, the cells were incubated with 2-[³H]deoxy-d-glucose for 10 min. The cell-associated radioactivity was measured by a scintillation counter. Values are means ± S.D. of triplicate determinations. B, the cells were pretreated with LY294002 and then stimulated with insulin and/or 10 μg/ml Et-3,4-dephostatin for 30 min.](https://doi.org/10.1074/jbc.R110.181324)

![FIG. 7. Effect of LY294002 on Et-3,4-dephostatin-induced GLUT4 translocation. 3T3-L1 adipocytes were treated with 10 nM insulin or 10 μg/ml Et-3,4-dephostatin without or with 100 μM LY294002 for 6 h, and then the cells were fixed with 2% formaldehyde. The fixed cells were stained with polyclonal anti-GLUT4 antibody that binds to the extracellular portion of GLUT4.](https://doi.org/10.1074/jbc.R110.181324)
induced intracellular c-Cbl tyrosine phosphorylation. After 100 
μm, LY294002 was pretreated for 30 min. 3T3-L1 adipocytes were 
treated with 10 nM insulin and/or 10 μg/ml Et-3,4-dephostatin for 6 h, 
and then the cells were lysed with the lysis buffer and immunoprecipi-
tated with monoclonal anti-c-Cbl antibody. Following electrophoretic 
transfer to the membranes, the proteins were immunoblotted with 
anti-phosphotyrosine antibody.

the addition of cycloheximide, the increase of hexose transport 
shown in Fig. 5 should not be due to the increase of glucose 
transporters. Vanadate was also reported to prolong insulin 
action by increasing the tyrosine phosphorylation of the insulin 
receptor (34). Vanadate activated PI 3-kinase-dependent glu-
cose transport (35). But Et-3,4-dephostatin appears more effec-
tive than vanadate, as is shown in Fig. 5B.

Unexpectedly, Et-3,4-dephostatin alone mimicked insulin 
action to induce several phenotypic changes. Compared with 
the quick action of insulin in 30 min, Et-3,4-dephostatin alone 
increased the tyrosine phosphorylation of the insulin receptor 
and IRS-1 rather slowly in 6 h, as shown in Fig. 2. This is 
because accumulation of phosphorylation by inhibition of 
PTPases takes time. Et-3,4-dephostatin strongly induced 
GLUT4 translocation and glucose transport. Therefore, Et-3,4- 
dephostatin may inhibit not only insulin receptor dephos-
phorylation but also dephosphorylation of other components 
that regulate glucose uptake. In fact, Et-3,4-dephostatin induced 
 tyrosine phosphorylation in several other proteins, including 
c-Cbl, PLCγ, PT 3-kinase, and IRS-3. In Fig. 2, Et-3,4-dephos-
tatin unexpectedly lowered the insulin-induced tyrosine phos-
phorylation of insulin receptor and IRS-1 in a 30-min incuba-
tion. Insulin is known to activate PTPases such as PTP-1B (36) 
that can dephosphorylate both insulin and IRS-1. Et-3,4-de-
phostatin might enhance induction of the inhibitor-insensitive 
PTPases with c-Cbl-associated protein at the site of caveola, and 
this protein complex up-regulates GLUT4 translocation 
through the PI 3-kinase independent pathway (21). Therefore, 
Et-3,4-dephostatin markedly enhanced tyrosine phosphoryla-
tion of c-Cbl, as shown in Fig. 8. Insulin-induced c-Cbl tyrosine 
phosphorylation greatly decreased at 6 h, but when Et-3,4-
dephostatin was added, the phosphorylation strongly re-
mained. Et-3,4-dephostatin alone also induced phosphorylation 
of c-Cbl. Thus, it is likely that Et-3,4-dephostatin potentiates 
both PI 3-kinase/Akt-dependent and PI 3-kinase/Akt- inde-
pendent pathways including activation of c-Cbl.

Insulin-induced glucose transport is mediated largely by PI 
3-kinase (37) and partly by p38 (38). On the other hand, the 
Ras/mitogen-activated protein kinase pathway and protein 
synthesis are not necessary in the effect of insulin. In Fig. 6A, 
insulin-induced glucose uptake was inhibited by LY294002 and 
SB203580 but not by a MEK inhibitor or by cycloheximide. 
Interestingly, LY294002 inhibited insulin-induced glucose 
transport and GLUT4 translocation completely in our assay 
system; but when Et-3,4-dephostatin was used, the inhibitor 
only partially suppressed glucose transport and GLUT4 trans-
location in a 6-h incubation (Fig. 6A and 7). On the other hand,
in a 30-min incubation, LY294002 completely blocked the in-
crease of glucose transport by Et-3,4-dephostatin, as shown in 
Fig. 6B. Therefore, it is likely that Et-3,4-dephostatin activates 
a PI 3-kinase-independent pathway in addition to the PI 3-ki-
nase/Akt pathway only in long term incubation. Very recently, 
Pessin and co-workers (21) showed the existence of a PI 3-ki-
nase-independent c-Cbl pathway in insulin-induced signal 
transduction. The proto-oncogenic product c-Cbl is known to be 
a component of protein-tyrosine kinase-mediated signaling cas-
cades downstream of the activated cell surface receptors. These 
receptors include the T cell, B cell, and cytokine receptors (39). 
The c-Cbl protein contains the RING finger domain, an exten-
sive proline-rich region that provides binding sites for the SH3 
domain, and the C-terminal leucine zipper domain that shows 
significant homology to ubiquitin-associated proteins (40). In 
hematopoietic cells, c-Cbl is known to be a negative regulator of 
Syk tyrosine kinase through the RING finger domain (41). In 
3T3-L1 cells, c-Cbl is prominently tyrosine-phosphorylated in 
response to insulin in 3T3-L1 adipocytes and not in 3T3-L1 
fibroblasts (42). The tyrosine-phosphorylated c-Cbl protein as-
soociates with Cbl-associated protein at the site of caveola, and 
this protein complex up-regulates GLUT4 translocation 
through the PI 3-kinase independent pathway (21). Therefore, 
it is possible that Et-3,4-dephostatin inhibits PTPases to pro-
tect phosphorylated c-Cbl from dephosphorylation. Actually, 
Et-3,4-dephostatin markedly enhanced tyrosine phosphoryla-
tion of c-Cbl, as shown in Fig. 8. Insulin-induced c-Cbl tyrosine 
phosphorylation greatly decreased at 6 h, but when Et-3,4-
dephostatin was added, the phosphorylation strongly re-
mained. Et-3,4-dephostatin alone also induced phosphorylation 
of c-Cbl. Thus, it is likely that Et-3,4-dephostatin potentiates 
both PI 3-kinase/Akt-dependent and PI 3-kinase/Akt- inde-
pendent pathways including activation of c-Cbl.

Kayali et al. (25) reported that phospholipase C-γ is associ-
ated with the insulin receptor and can be phosphorylated by 
the receptor. Therefore, if a PI 3-kinase is not involved down-
stream of phospholipase C-γ, activation of phospholipase C-γ 
by inhibition of dephosphorylation may also be a possible 
mechanism.

When Et-3,4-dephostatin was orally given to mice with high 
blood glucose, it lowered their blood glucose significantly, as 
shown in Fig. 9. Therefore, Et-3,4-dephostatin is likely to be 
absorbed from the intestine and to be stable in the body. Dur-
ing the in vivo experiment, no toxicity, including the loss of 
blood weight, was observed. In 24 h, there was no marked 
difference in food uptake between the test and control group.

FIG. 8. Effect of LY294002 on insulin or Et-3,4-dephostatin-
induced intracellular c-Cbl tyrosine phosphorylation. After 100 
μm, LY294002 was pretreated for 30 min. 3T3-L1 adipocytes were 
treated with 10 nM insulin and/or 10 μg/ml Et-3,4-dephostatin for 6 h, 
and then the cells were lysed with the lysis buffer and immunoprecipi-
tated with monoclonal anti-c-Cbl antibody. Following electrophoretic 
transfer to the membranes, the proteins were immunoblotted with 
anti-phosphotyrosine antibody.

FIG. 9. Decrease in blood glucose level in Et-3,4-dephostatin-
treated KK-Ay mice. KK-Ay mice were treated with a CE-2 diet 
containing 12% sucrose and Et-3,4-dephostatin for 24 h. A blood sample 
was then taken from the orbital capillary bed, and its glucose level was 
measured. The results are representative of two independent experiments.
fece. The methoxime-type derivative of dephostatin (29) belongs to the PTPase inhibitors of the second generation. Long term experiments with this compound also decreased the blood glucose level in mice without toxicity. Thiazolidine compounds are being widely used clinically for the treatment of type 2 diabetes mellitus. Its target is known to be peroxisome proliferator-activated receptor γ, which is a unique transcription factor for adipocyte differentiation. In our assay system shown under “Experimental Procedures,” troglitazone significantly enhanced differentiation of 3T3-L1 cells into adipocytes. However, Et-3,4-dephostatin did not increase the differentiation significantly (data not shown). Therefore, the mechanism of the antidiabetic effect by Et-3,4-dephostatin in vivo should be different from that by troglitazone, and its side effects may not include obesity, unlike the case for troglitazone.

Thus, Et-3,4-dephostatin potentiated the insulin-related signal transduction in cultured 3T3-L1 adipocytes and showed antidiabetic effects in mice. Especially, it was orally active in vivo. Since the nitrosamine group in Et-3,4-dephostatin may be mutagenic and carcinogenic, a nitrosamine-free analogue of Et-3,4-dephostatin (29) may be a prototype of new antidiabetic agents.

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