Biochemical Pharmacology, Vol. 51, pp. 1061–1067, 1996.
Copyright © 1996 Elsevier Science Inc.

ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02438-7

Pervanadate Stimulation of Wortmannin-Sensitive and -Resistant 2-Deoxyglucose Transport in Adipocytes

*Motoko Ida, Kiyoshi Imai, Seiichi Hashimoto and Hiroyuki Kawashima**

MOLECULAR MEDICINE RESEARCH LABORATORIES, YAMANOUCHI INSTITUTE FOR DRUG DISCOVERY RESEARCH, YAMANOUCHI PHARMACEUTICAL CO., LTD., IBARAKI 305, JAPAN

ABSTRACT. Pervanadate mimics several distinct insulin effects, including stimulation of hexose uptake in the *in vitro* system, and reduces the blood glucose level in streptozotocin-treated diabetic rats. It has been proposed that pervanadate induces insulin-like effects mediated through autophosphorylation and activation of insulin receptor (IR) even in the absence of insulin by inhibiting protein tyrosine phosphatases. This study focused on the mechanism of pervanadate action on hexose uptake. Both insulin (100 nM) and pervanadate (100 μM), a protein tyrosine phosphatase inhibitor, induced a marked increase in the phosphorylation at tyrosine residues of IR and insulin receptor substrate 1 (IRS-1) and in 2-deoxyglucose uptake in 3T3-L1 adipocytes. Wortmannin (1 μM), a specific phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor, inhibited the increased 2-deoxyglucose uptake by insulin completely but that by pervanadate only partially. On the other hand, both insulin- and pervanadate-stimulated PI 3-kinase activities were inhibited completely by wortmannin (100 nM), suggesting that the pervanadate-induced wortmannin-resistant effect on hexose uptake may be mediated through a PI 3-kinase-independent pathway. This pervanadate-induced wortmannin-resistant effect was abolished by ST-638, a specific tyrosine kinase inhibitor. These data suggest that at least two distinct tyrosine phosphorylation pathways may be involved in the insulin-like effect of pervanadate. BIOCHEM PHARMACOL 51;8:1061–1067, 1996.

KEY WORDS. glucose uptake; adipocytes; wortmannin; tyrosine phosphorylation; insulin receptor; IRS-1

Insulin produces a variety of physiological effects on its target cells. These include stimulation of glucose uptake, lipogenesis, protein synthesis, and inhibition of lipolysis. The first step in the signal transduction pathway of insulin action is the binding of insulin to IR,† which leads to autophosphorylation and activation of IR tyrosine kinase. Tyrosine-phosphorylated IR then phosphorylates IRS-1 [1], which, in turn, promotes the association of PI 3-kinase to IRS-1, resulting in an activation of PI 3-kinase [2]. This is mediated through binding of SH2 domains of p85 regulatory subunit of PI 3-kinase to tyrosine phosphorylation sites in YXXM motifs of IRS-1. The degree of phosphorylation on tyrosine residues of IRS-1 it thought to determine the degree of cellular response to insulin. It has been reported recently, that the effects of insulin to stimulate glucose uptake and to antagonize cyclic AMP-mediated lipolysis in rat adipocytes are abolished by wortmannin, a specific inhibitor of PI 3-kinase, and the observations indicate that PI 3-kinase plays a role in these responses to insulin [3].

Pervanadate, a potent inhibitor of protein tyrosine phosphatase, mimics those insulin effects in rat adipocytes [4, 5] and reduces the blood glucose level in streptozotocin-treated diabetic rats [6] possibly through inhibition of protein tyrosine phosphatase, thereby permitting tyrosine phosphorylation and activation of IR kinase [4, 5, 7–9]. It has also been reported that pervanadate induces tyrosine phosphorylation of IRS-1 and activation of PI 3-kinase upon its association with IRS-1 in intact rat livers [10]. The pervanadate-induced stimulation of cellular hexose uptake, therefore, could also be mediated through an activation of PI 3-kinase. However, pervanadate is not selective for any particular tyrosine phosphatase, and thus its insulin-like effects may not necessarily be abolished by wortmannin. To further define the role of PI 3-kinase in the pervanadate-induced stimulation of hexose transport, the effects of wortmannin and of ST-638 [11, 12], a tyrosine kinase inhibitor, were tested. ST-638 can inhibit tyrosine kinases like EGF receptor kinase, p70*gag-actin-v-fgr* kinase, pp60*c-src* kinase, p130*gag-v-fps* kinase, and pp60*v-src* kinase with IC_{50} values of 1.1, 4.2, 18, 70, and 87 μM, respectively, but has no inhibitory effect on serine/threonine kinases including

* Corresponding author: Dr. Hiroyuki Kawashima, Molecular Medicine Research Laboratories, Yamanaouchi Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305, Japan. Tel. 81-298-52-5111; FAX 81-298-52-2925.

Received 3 May 1995; accepted 16 November 1995.

† *Abbreviations*: IR, insulin receptor; IRS-1, insulin receptor substrate-1; EGF, epidermal growth factor; PBS(−), PBS without magnesium and calcium; PI 3-kinase, phosphatidylinositol 3-kinase; DMEM, Dulbecco's modified Eagle's medium; and V/H, a combination of vanadate and H_2O_2.

cAMP-dependent protein kinase and protein kinase C at concentrations up to 100 μM [12]. ST-638 has a structure similar to that of tyrosine residues in the substrate protein and can competitively inhibit the phosphorylation of an exogenous substrate by EGF receptor kinase and p130*gag-v-fps* kinase [11, 12].

We report here that cellular 2-deoxyglucose uptake, stimulated by VH at lower concentrations, was inhibited completely by wortmannin, whereas at higher concentrations it was inhibited only partially and the remaining activity was abolished by the simultaneous presence of ST-638.

MATERIALS AND METHODS

Materials

Sodium orthovanadate, dexamethasone, 3-isobutyl 1-methylxanthine (IBMX) and 2-deoxy-D-glucose were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). H_2O_2 and a protein assay kit were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Insulin and wortmannin were obtained from Biomedical Technologies Inc. (Stoughton, MA, U.S.A.) and the Kyowa Medex Co., Ltd. (Tokyo, Japan), respectively. ST-638 was provided by the Kanegafuchi Chemical Industry Co. Ltd. (Takasago, Japan). 2-Deoxy-D-[1-^{14}C]glucose and enhanced chemiluminescence (ECL) reagents were purchased from the Amersham Corp. (Arlington Heights, IL, U.S.A.). Antibodies against phosphotyrosine (4G10) and IRS-1 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Antibodies against mouse IgG1 and mitogen-activated protein (MAP) kinase were from Zymed (San Francisco, CA, U.S.A.). Anti-insulin receptor antibody was from Oncogene Science Inc. (Uniondale, NY, U.S.A.). SDS–PAGE molecular weight standards were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). LY294002 was a gift from Dr. Masuyama in the Cardiovascular & Atherosclerosis Research Laboratory of the Yamanouchi Pharmaceutical Co. Ltd.

Cell Culture

3T3-L1 preadipocytes were cultured with DMEM supplemented with 10% fetal bovine serum in either 100-mm culture dishes or 6-well culture plates in a humidified atmosphere containing 95% air and 5% CO_2 at 37° and induced to differentiate into adipocytes as described by Rubin *et al.* [13] with minor modification. After the preadipocytes reached confluence, differentiation into adipocytes was induced by incubating the cells for 2 days in medium containing 0.5 mM IBMX, 0.25 μM dexamethasone and 10 μg/mL insulin. The cells were then incubated in medium containing 10 μg/mL insulin with medium changes every 2–3 days for an additional 6–10 days. Thereafter, the cells were maintained in medium without insulin for 2 days.

Preparation of Extracts from 3T3-L1 Adipocytes Treated without or with Insulin or V/H

Pervanadate was prepared by mixing equimolar concentrations of sodium orthovanadate and H_2O_2 (V/H), followed by incubation for 15 min at room temperature. When specifically described as pervanadate but not V/H in the text, pervanadate was prepared by addition of catalase 15 min after mixing of orthovanadate and peroxide to degrade the excess of peroxide. 3T3-L1 adipocytes in 100-mm dishes were incubated in DMEM without serum overnight and then treated without or with insulin or V/H for the indicated time periods. After washing the cells with ice-cold PBS(−), the cells were lysed in 1 mL of lysis buffer A (20 mM Tris, pH 7.4, 1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 2 mM $N_{a3}VO_4$, 10 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 12,000 *g* for 10 min. The supernatants were subjected to immunoprecipitation of IR, IRS-1, or tyrosine-phosphorylated proteins with specific antibodies, or were diluted with equal volume of two times concentrated Laemmli sample buffer for SDS–PAGE.

Immunoprecipitation of IR, IRS-1, and Tyrosine-Phosphorylated Proteins from 3T3-L1 Adipocyte Extracts

IR, IRS-1, and tyrosine-phosphorylated proteins in the cell extracts were immunocomplexed with anti-IR antibody, anti-IRS-1 antibody, and anti-phosphotyrosine antibody (4G10), respectively. These immune complexes were precipitated by binding to protein A-agarose and then centrifuged at 12,000 *g* for 5 min. Immune complexes that bound to protein A-agarose beads were washed three times with lysis buffer. Immune complexes with anti-IR antibody and anti-IRS-1 antibody were solubilized in 120 μL of Laemmli sample buffer. Immune complexes with anti-phosphotyrosine antibody were subjected to determination of PI 3-kinase activity.

Immunoblotting

After being boiled, samples were subjected to SDS–PAGE (8%) [14]. The separated proteins were electrotransferred to a nitrocellulose membrane at 2.5 mA/cm^2 for 1 hr. After blocking in 5% skim milk, the blot was incubated with anti-phosphotyrosine antibody. After washing, the blot was developed with horseradish peroxidase conjugated to rabbit anti-mouse IgG antibody for detection of tyrosine-phosphorylated proteins. The ECL reagents were then used to detect immunoreactive proteins.

Measurement of 2-Deoxyglucose Uptake

3T3-L1 adipocytes cultured in 6-well plates were incubated overnight in DMEM without serum, washed three times with PBS(−), and then the medium was replaced with Krebs-Ringer-phosphate (KRP). Cells were treated without

or with insulin, V/H, wortmannin, and/or ST-638 for 30 min at 37°. 2-Deoxyglucose containing 1-^{14}C-labeled tracer (0.5 mCi/mmol) was added to the culture at a final concentration of 0.2 mM. The incubation was continued for an additional 10 min at 37°, and then terminated by the addition of ice-cold PBS(−). The cells were immediately washed three times with ice-cold PBS(−) and were then solubilized in 2 mL of 0.5% SDS. The radioactivity in 0.5 mL was determined in a scintillation counter in 5 mL Aquasol-2 fluid. Ten microliters of the lysates was used for protein assay.

Determination of the PI 3-Kinase Activity

3T3-L1 adipocytes cultured in 100-mm dishes were treated without or with insulin (100 nM) or various concentrations of V/H for 15 min at 37°. Preparation of cell extracts and immunoprecipitation with anti-phosphotyrosine antibody (4G10) were performed as described above. Immune complexes that bound to protein A-agarose beads were further washed once with 50 mM HEPES buffer, pH 7.5, twice with buffer B (20 mM Tris, pH 7.5, 100 mM NaCl and 0.5 mM EGTA), and assayed for PI 3-kinase activities. PI 3-kinase activities in the immune complexes were determined in 50 μL of the reaction mixture consisting of 20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 3.5 mM $MgCl_2$, 0.2 mg/mL phosphatidylinositol and 43 μM (4.2 nCi/μL) [γ-^{32}P]ATP in the absence or presence of 100 nM wortmannin. The reaction was carried out at 25° for 10 min and terminated by the addition of 100 μL of 1 N HCl and 200 μL of chloroform:methanol (1:1). After vigorous stirring and subsequent centrifugation, the upper layer was discarded carefully and the lower layer was added with 80 μL of 1 N HCl:MeOH (1:1). The mixture was stirred vigorously and then centrifuged. Next 60 μL of the organic phase was evaporated to dryness, dissolved in 30 μL of $CHCl_3$:MeOH (4:1), and spotted on a silica gel 60 plate. The plate was developed in $CHCl_3$:CH_3, OH:H_2O:NH_4OH (75:100:15:25). The dried plate was visualized for the radioactivities with a Fuji BAS 2000 Bioimaging Analyzer.

RESULTS AND DISCUSSION

Effect of Wortmannin on Insulin- or V/H-Induced Stimulation of 2-Deoxyglucose Uptake in 3T3-L1 Adipocytes

Treatment of 3T3-L1 adipocytes with insulin (100 nM) induced a marked increase in cellular 2-deoxyglucose uptake (Fig. 1). The maximum response was obtained at 100 nM (data not shown). Treatment of the cells with V/H, which generates pervanadate, a powerful inhibitor of protein tyrosine phosphatase, also induced a stimulation of 2-deoxyglucose uptake in a concentration-dependent manner. This effect of V/H was detectable at concentrations as low as 10 μM and reached maximum at 50 μM (Fig. 1). Treatment of 3T3-L1 adipocytes with wortmannin, an inhibitor of PI 3-kinase, alone decreased cellular hexose transport below the basal level (Fig. 1). Insulin-induced stimulation of 2-deoxyglucose uptake was blocked completely by the simultaneous treatment of the cells with wortmannin at concentrations as low as 100 nM (Fig. 2). This is consistent with the observation reported previously [3]. The V/H-induced stimulation at lower concentrations (10–20 μM) also was blocked completely by wortmannin (1 μM) (Fig. 1). However, when higher concentrations of V/H were used for the stimulation, wortmannin treatment (1 μM) failed to abolish the effect of V/H. This wortmannin-resistant effect of V/H was detectable at a concentration of 30 μM and was concentration dependent over 30–300 μM. When 300 μM V/H was used for the stimulation, about 60% of the V/H-induced increase in the hexose up-

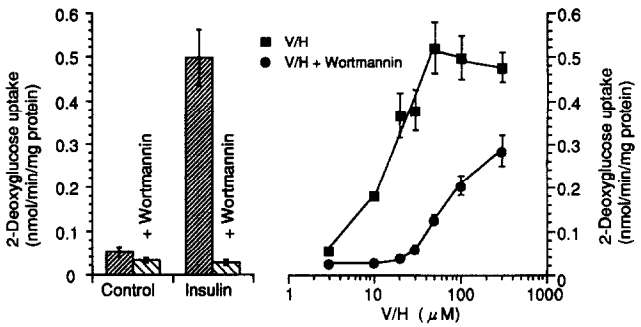

FIG. 1. Effect of wortmannin on insulin- or V/H-induced stimulation of 2-deoxyglucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. The cells were treated without (Control) or with insulin (100 nM) (Insulin), V/H (3–300 μM) (V/H), and/or wortmannin (1 μM) for 30 min. Cellular hexose uptake during a further 10-min incubation with the addition of [^{14}C]2-deoxyglucose was determined as described under Materials and Methods. Data are means ± SEM, N = 6. Where error bars are not shown, the bars were smaller than the symbol.

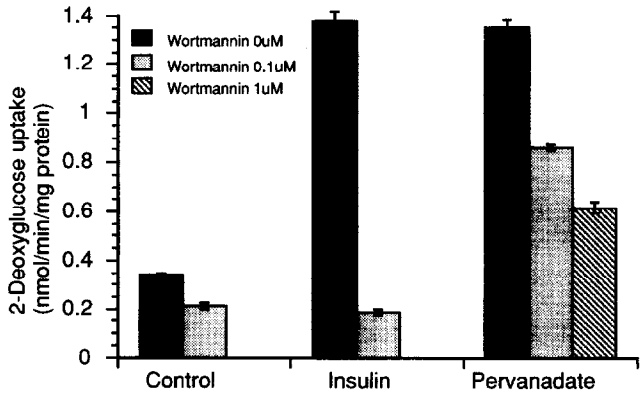

FIG. 2. Effect of wortmannin on pervanadate-induced stimulation of 2-deoxyglucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. The cells were treated without or with insulin (100 nM), pervanadate (100 μM), and/or wortmannin (100 nM or 1 μM) for 30 min. Cellular hexose uptake was determined as described in the legend of Fig. 1. Data are means ± SEM, N = 6.

take still remained even in the presence of wortmannin at 1 μM (Fig. 1). This observation suggests that V/H at higher concentrations (≥30μM) activates not only wortmannin-sensitive but also wortmannin-resistant pathways, both of which stimulate cellular hexose transport in the cells. To elucidate whether the effect of V/H on hexose uptake is due for the most part, to the effect of pervanadate but not that of H_2O_2 or orthovanadate, 3T3-L1 adipocytes were treated with pervanadate (100 μM), which was prepared by the addition of catalase 15 min after the mixing of orthovanadate and H_2O_2, or with orthovanadate (100 μM–3 mM), and then 2-deoxyglucose uptake was determined. When pervanadate was used for the stimulation of 3T3-L1 adipocytes, 2-deoxyglucose uptake increased to a level comparable to that induced by insulin, and 1 μM wortmannin treatment again failed to abolish the effect of pervanadate (Fig. 2). The degree of and the wortmannin-resistant portion of the increase in 2-deoxyglucose uptake induced by pervanadate were similar to that by V/H (Figs. 1 and 2). On the other hand, orthovanadate at concentrations from 100 μM up to 3 mM induced only a slight increase (24–71%) in 2-deoxyglucose uptake (data not shown), which is much less than the increases induced by insulin, pervanadate, or V/H (~10-fold) (Figs. 1 and 2). These observations strongly indicate that the V/H-induced increase in cellular 2-deoxyglucose uptake, both wortmannin-sensitive and -resistant, is elicited by pervanadate. Moreover, the degree of the inhibition of V/H-stimulated 2-deoxyglucose uptake by wortmannin at 10 μM was not much different from that at 100 nM (Fig. 3), which suggests that the wortmannin-resistant 2-deoxyglucose uptake may not be due to the perturbing effect by V/H on the permeation of wortmannin into the cells.

To further examine whether the wortmannin-resistant portion of V/H-stimulated 2-deoxyglucose uptake is independent of PI 3-kinase, we used another specific PI 3-kinase inhibitor, LY294002. The insulin-induced stimulation of 2-deoxyglucose uptake was abolished by the simultaneous treatment of the cells with LY294002 at a concentration of 30 μM (Fig. 4). However, 30 μM LY294002 also failed to abolish the effect of pervanadate on 2-deoxyglucose uptake (Fig. 4). This result suggests that pervanadate-induced stimulation of hexose transport is mediated by at least two pathways: one is PI 3-kinase inhibitor sensitive and the other is PI 3-kinase inhibitor resistant.

Exposure of the cells to insulin (100 nM) plus V/H (100 μM) did not cause any further increase in 2-deoxyglucose uptake above the level reached with either insulin or V/H alone (Fig. 5). These observations suggest that both wortmannin-sensitive and -resistant pathways may share a common step, probably proximal to a translocation of glucose transporters from low density microsomes to plasma membrane.

Effect of Wortmannin on Tyrosine Phosphorylation of Cellular Proteins and PI 3-Kinase Activity Stimulated by Insulin and V/H in 3T3-L1 Adipocytes

Exposure of 3T3-L1 adipocytes to insulin (100 nM) markedly increased not only phosphorylation at tyrosine residues of specific cellular proteins including IR and IRS-1 (Fig. 6) but also PI 3-kinase activity in the immunoprecipitates with anti-phosphotyrosine antibody (Fig. 7). V/H mimicked these effects of insulin in a concentration-dependent manner, and all the V/H-induced insulin-like effects on hexose transport, tyrosine phosphorylation of IR and IRS-1, and PI 3-kinase activity were detectable at concentrations as low as 10 μM (Figs. 1, 6 and 7), suggesting that at this concentration these insulin-like effects may be mediated through tyrosine phosphorylation and activation of IR. Pre-

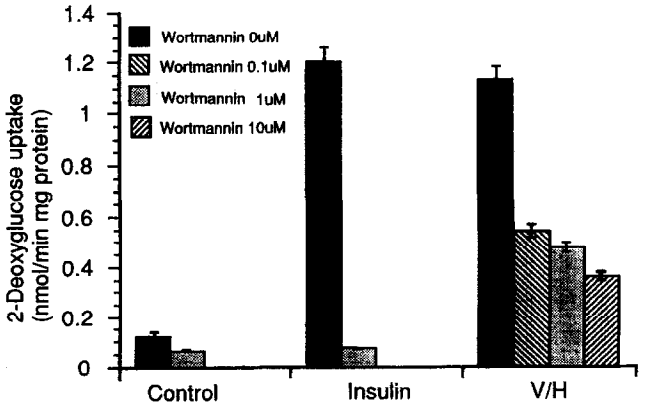

FIG. 3 Effect of increasing concentrations of wortmannin on V/H-induced stimulation of 2-deoxyglucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. The cells were treated without or with insulin (100 nM), V/H (100 μM), and/or increasing concentrations of wortmannin (100 nM–10 μM) for 30 min. Cellular hexose uptake was determined as described in the legend of Fig. 1. Data are means ± SEM, N = 6.

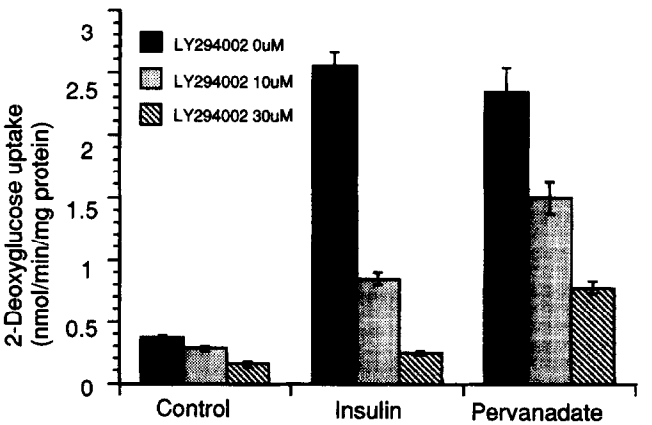

FIG. 4. Effect of LY294002 on insulin- or pervanadate-induced stimulation of 2-deoxyglucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. The cells were treated without (Control) or with insulin (100 nM), pervanadate (100 μM), and/or LY294002 (10 and 30 μM) for 30 min. The cellular hexose uptake was determined as described in Fig. 1. Data are means ± SEM, N = 3.

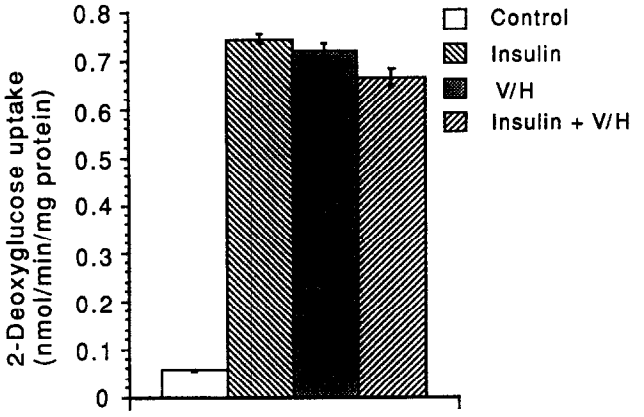

FIG. 5. Effects of insulin, V/H, and insulin plus V/H on 2-deoxyglucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. The cells were treated without (Control) or with insulin (100 nM) (Insulin), V/H (100 μM) (V/H), or insulin (100 nM) plus V/H (100 μM) (insulin + V/H). Cellular hexose uptake was determined as described in the legend of Fig. 1. Data are means ± SEM, N = 6.

treatment of the cells with wortmannin (1 μM) did not prevent the insulin- and V/H-induced tyrosine phosphorylation of IR and IRS-1 (Fig. 8A). On the other hand, wortmannin (100 nM) completely inhibited PI 3-kinase activity stimulated by insulin as well as V/H (Fig. 7). These data

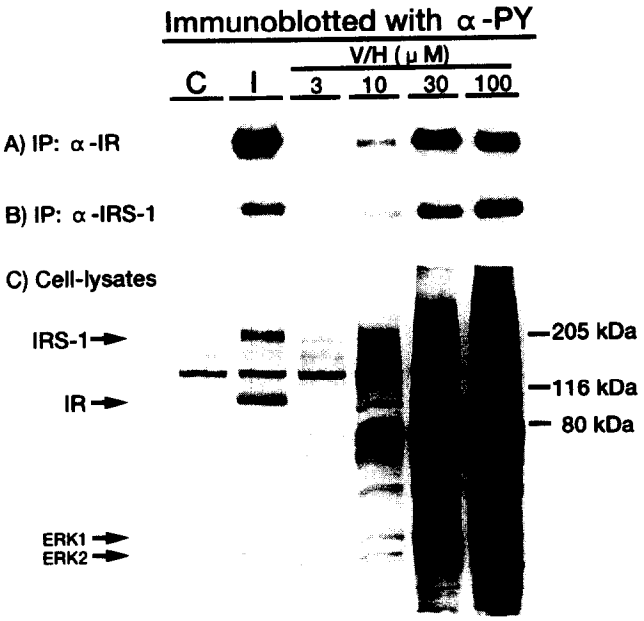

FIG. 6. Insulin- or V/H-induced increase in the phosphorylation of cellular proteins at tyrosine residues. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. The cells were treated without (C) or with insulin (100 nM) (I) or V/H (3–100 μM) (V/H) for 15 min. Cell-lysates and immunoprecipitates of IR and IRS-1 were prepared as described under Materials and Methods. Aliquots of the samples were immunoblotted for phosphotyrosine. Abbreviations: α-py, antibody against phosphotyrosine (4 G 10); ERK1 and ERK2, extracellular signal-regulated protein kinase 1 and 2, respectively.

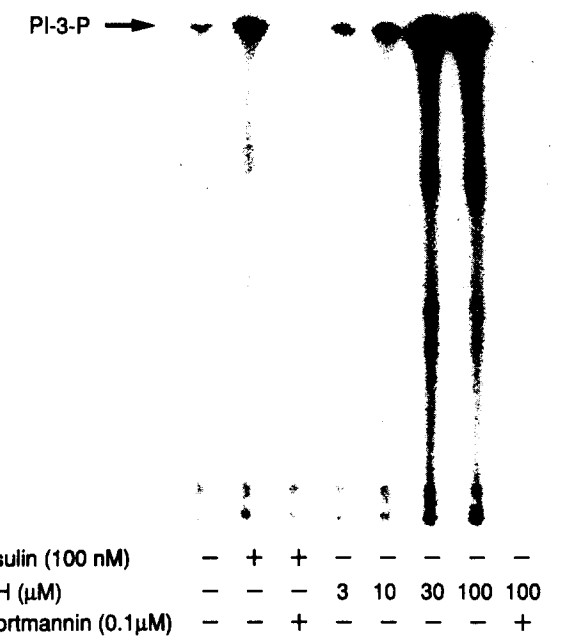

FIG. 7. Effect of wortmannin on insulin- or V/H-stimulated PI 3-kinase activity in the immune complexes with anti-phosphotyrosine antibody. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. The cells were treated without or with insulin (100 nM) or V/H (3–100 μM) for 15 min. The PI 3-kinase activity in the immune complexes with anti-phosphotyrosine antibody was determined in the absence or presence of 100 nM wortmannin.

suggest that V/H, at lower concentrations, stimulates hexose transport possibly through the same mechanism of insulin action, e.g. sequential tyrosine phosphorylation of IR and IRS-1 which, in turn, activates PI 3-kinase, followed by translocation of glucose transporter to plasma membrane, leading to stimulation of cellular glucose uptake. By contrast, wortmannin failed to abolish hexose uptake induced by higher concentrations (≥30 μM) of V/H (Fig. 1). This may be explained by the fact that V/H, at higher concentrations, increased the level of tyrosine phosporylation not only of IR and IRS-1 but also of many other cellular proteins (Figs. 6 and 8A), some of which may be involved in the wortmannin-resistant hexose uptake. This possibility can be tested by comparing the extent of tyrosine phosphorylation on these proteins and the wortmannin-resistant hexose transport activity, both of which are stimulated by V/H. Therefore, we next examined the effect of tyrosine kinase inhibitor on these two effects of V/H.

Effect of ST-638 on Cellular 2-Deoxyglucose Uptake and Tyrosine Phosphorylation of Cellular Proteins Stimulated by Insulin and V/H

It has been reported that pervanadate stimulation of HL-60 cells at a concentration of 50 μM induces the phosphorylation of a number of cellular proteins and phosphatidylethanol formation through activation of phospholipase

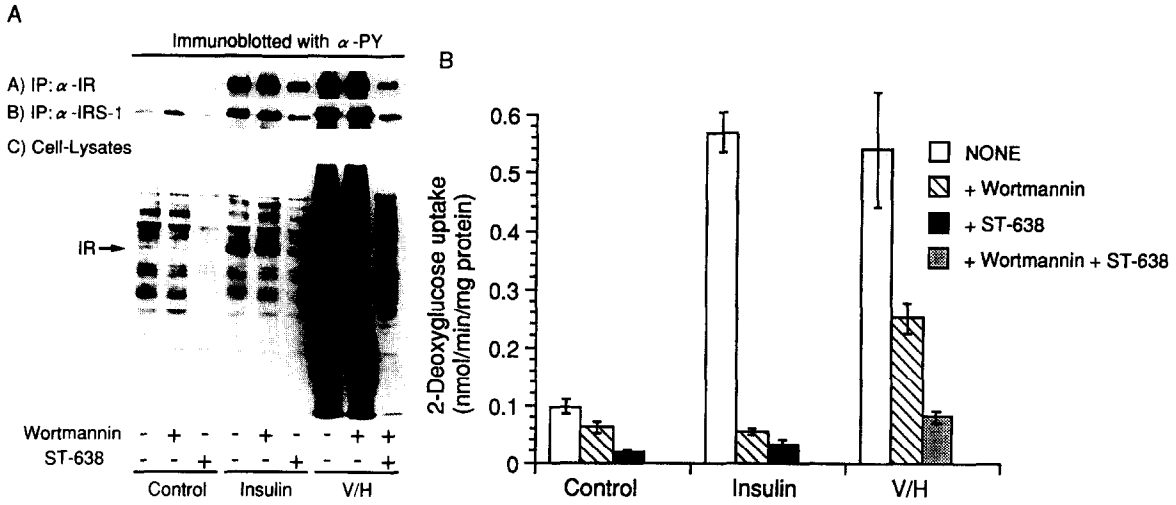

FIG. 8. Effect of ST-638 or wortmannin on insulin- or V/H-induced increase in the phosphorylation of cellular proteins at tyrosine residues and in the 2-deoxyglucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes in culture were deprived of serum for 16 hr before treatment. In panel A, the cells were pretreated without or with ST-638 (300 μM) and/or wortmannin (1 μM) for 30 min and then treated without (Control) or with insulin (100 nM), (Insulin) or V/H (100 μM) (V/H) for 15 min. Cell-lysates and immunoprecipitates of IR and IRS-1 were prepared as described under Materials and Methods. Aliquots of the samples were immunoblotted for phosphotyrosine. In panel B, the cells were treated without (Control) or with insulin (100 nM) (Insulin), V/H (100 μM) (V/H), wortmannin (1 μM), and/or ST-638 (300 μM) for 30 min. Cellular hexose uptake was determined as described in the legend of Fig. 1. Data are means ± SEM, N = 6.

D, which were reduced by pretreatment with 100 μM ST-638, a specific tyrosine kinase inhibitor, by 71.7 ± 8.6 and 88.5 ± 8%, respectively [15]. In our experiments to examine the effects of V/H on hexose uptake and protein tyrosine phosphorylation, we used 100 μM V/H and 300 μM ST-638, respectively. With the dosage of ST-638 chosen, almost complete inhibition of tyrosine phosphorylation may be expected.

As depicted in Fig. 8A, insulin (100 nM) induced a marked increase in the phosphorylation at tyrosine residues of specific cellular proteins including IR and IRS-1 in 3T3-L1 adipocytes, and this was reduced dramatically by ST-638. The insulin-induced 2-deoxyglucose uptake by the cells was also abolished by ST-638 (Fig. 8B). As previously reported [5], V/H (100 μM) not only mimicked the effect of insulin on tyrosine phosphorylation, but also induced a marked tyrosine phosphorylation of a number of cellular proteins other than those induced by insulin (Fig. 8A). The levels of tyrosine phosphorylation of IR and IRS-1 induced by V/H (100 μM) were comparable to those induced by insulin (100 nM), and were equally suppressed by ST-638 (Fig. 8A). The phosphorylation of proteins induced by V/H but not by insulin was also suppressed by ST-638 (Fig. 8A). In addition, ST-638 almost completely suppressed the wortmannin-resistant portion of the V/H-induced 2-deoxyglucose uptake (Fig. 8B). Since the maximum stimulation of 2-deoxyglucose transport by V/H was comparable to that by insulin, and since the effects of these agents were not additive (Fig. 5), the mechanisms of action by which these agents stimulate glucose transport could not be independent. On the other hand, wortmannin should have abolished the V/H effect on hexose uptake, provided there would be only one signalling pathway downstream of PI 3-kinase leading to activation of cellular hexose uptake. Thus, suppression of ST-638 of the V/H-induced wortmannin-resistant hexose uptake (Fig. 8B) may be mediated through inhibition of protein tyrosine phosphorylation of cellular protein(s), which is (are) distinct from IR tyrosine kinase and IRS-1. Taken together, these data and considerations clearly indicate that there are at least two signalling pathways for pervanadate-inducible cellular hexose uptake. One is the well-established pathway (pathway I) beginning from sequential phosphorylation of IR and IRS-1 followed by activation of PI 3-kinase through the final step of hexose transport. The other (pathway II) involves tyrosine kinase(s), which is (are) distinct from and independent of those involved in pathway I. Pathway II must join pathway I somewhere downstream of PI 3-kinase and share a common pathway thereafter to the translocation of glucose transporters from low density microsomes to plasma membrane, since no additive effect was observed for wortmannin-sensitive and -resistant hexose uptake (Fig. 5). It is not known at present whether this wortmannin-resistant pathway has any biological significance in glucose metabolism, nor is it clear what is a ligand or factor involved in this pathway. These are subjects for future study that may provide new information on the regulatory mechanisms underlying glucose metabolism and insulin action.

In conclusion, we demonstrated that V/H stimulates cellular hexose transport at lower concentrations (10–20 μM)

through the wortmannin-sensitive pathway, and at higher concentrations (≥30 μM) through both wortmannin-sensitive and -resistant pathways in 3T3-L1 adipocytes.

We thank Dr. Koyo Matduda at the Cardiovascular and Atherosclerosis Research Laboratories, Yamanouchi Institute for Drug Discovery Research, for the chemical synthesis of LY294002.

References

1. Sun XJ, Miralpeix M, Myers MG, Glasheen EM, Backer JM, Kahn CR and White MF, Expression and function of IRS-1 in insulin signal transmission. *J Biol Chem* **267:** 22662–22672, 1992.
2. Backer JM, Myers MG, Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J and White MF, Phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J* **11:** 3469–3479, 1992.
3. Okada T, Kawano Y, Sakakibara T, Hazeki O and Ui M, Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes: Studies with a selective inhibitor wortmannin. *J Biol Chem* **269:** 3568–3573, 1994.
4. Fantus IG, Kadota S, Deragon G, Foster B and Posner BI, Pervanadate [peroxide(s) of vanadate] mimics insulin action in rat adipocytes via activation of the insulin receptor tyrosine kinase. *Biochemistry* **28:** 8864–8871, 1989.
5. Shisheva A and Shechter Y, Mechanism of pervanadate stimulation and potentiation of insulin-activated glucose transport in rat adipocytes: Dissociation from vanadate effect. *Endocrinology* **133:** 1562–1568, 1993.
6. Shisheva A, Ikonomov O and Shechter Y, The protein tyrosine phosphatase inhibitor, pervanadate, is a powerful antidiabetic agent in streptozotocin-treated diabetic rats. *Endocrinology* **134:** 507–510, 1994.
7. Kadota S, Fantus IG, Deragon G, Guyda HJ, Hersh B and Posner BI, Peroxide(s) of vanadium: A novel and potent insulin-mimetic agent which activates the insulin receptor kinase. *Biochem Biophys Res Commun* **147:** 259–266, 1987.
8. Kadota S, Fantus IG, Deragon G, Guyda HJ and Posner BI, Stimulation of insulin-like growth factor II receptor binding and insulin receptor kinase activity in rat adipocytes. *J Biol Chem* **262:** 8252–8256, 1987.
9. Heffetz D, Rutter WJ and Zick Y, The insulinomimetic agents H_2O_2 and vanadate stimulate tyrosine phosphorylation of potential target proteins for the insulin receptor kinase in intact cells. *Biochem J* **288:** 631–635, 1992.
10. Hadari YR, Tzahar E, Nadiv O, Rothenberg P, Roberts CT, LeRoith D, Yarden Y and Zick Y, Insulin and insulinomimetic agents induce activation of phosphatidylinositol 3′-kinase upon its association with pp185 (IRS-1) in intact rat livers. *J Biol Chem* **267:** 17483–17486, 1992.
11. Shiraishi T, Domoto T, Imai N, Shimada Y and Watanabe K, Specific inhibitors of tyrosine-specific protein kinase, synthetic 4-hydroxycinnamide derivatives. *Biochem Biophys Res Commun* **147:** 322–328, 1987.
12. Shiraishi T, Owada MK, Tatsuka M, Yamashita T, Watanabe K and Kakunaga T, Specific inhibitors of tyrosine-specific protein kinases: Properties of 4-hydroxycinnamide derivatives *in vitro*. *Cancer Res* **49:** 2374–2378, 1989.
13. Rubin CS, Hirsch A, Fung C and Rosen OM, Development of hormone receptors and hormonal responsiveness *in vitro*: Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. *J Biol Chem* **253:** 7570–7578, 1978.
14. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
15. Bourgoin S and Grinstein S, Peroxides of vanadate induce activation of phospholipase D in HL-60 cells. *J Biol Chem* **267:** 11908–11916, 1992.