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Bis(*N*,*N*-dimethylhydroxamido)hydroxooxovanadate Inhibition of Protein Tyrosine Phosphatase Activity in Intact Cells

COMPARISON WITH VANADATE

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ABSTRACT. We have shown previously that bis(*N*,*N*-dimethylhydroxamido)hydroxooxovanadate (DMHV) is an excellent reversible inhibitor of protein tyrosine phosphatase (PTP) *in vitro*. DMHV does not carry a charge under physiological pH conditions and is anticipated to permeate cell membranes more easily than vanadate. In the present study, the efficacy of DMHV as a PTP inhibitor in intact cells was compared with that of vanadate by measuring phosphotyrosine levels in various cells treated with these compounds. DMHV was more effective in increasing both the phosphotyrosine levels of various proteins in 3T3L1 fibroblasts and the level of insulin-receptor phosphorylation in CHO cells overexpressing the human insulin receptor. DMHV was about 10- to 20-fold more effective than vanadate in increasing glucose transport and glycogen synthesis in 3T3L1 adipocytes. DMHV, unlike vanadate, also inhibited PTP in Jurkat cells. The implications of these observations are discussed. BIOCHEM PHARMACOL **58**;12:1859–1867, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. protein tyrosine phosphatase; insulin action; vanadate; dimethylhydroxamidohydroxo-oxovanadate; glucose transport; glycogen synthesis

Reversible phosphorylation on tyrosine residues of proteins plays an essential role in the regulation of a wide variety of cellular activities. The phosphorylation of protein on tyrosine is controlled both by the concerted action of PTK that transfer the terminal phosphate from nucleoside triphosphate to tyrosine on the protein and by PTP that remove phosphate from phosphotyrosine-containing proteins [see Ref. 1 for review]. Tyrosine phosphorylation levels are under dynamic control, and the levels in the cell reflect the balance of the activities of the PTK and PTP acting on the individual protein.

PTP form a large family of enzymes that can be broadly classified into either cytosolic or transmembrane types [see Refs. 2–4 for reviews]. The cytosolic enzymes have a single catalytic domain of about 240 amino acids and various regulatory domains on either the amino- or carboxy-terminal ends that appear to play a role in the localization and/or regulation of catalytic activity. The transmembrane type of enzyme has one or two homologous catalytic domains linked by a transmembrane domain to an extracellular domain of varying length. It has been suggested that the extracellular domain may regulate the catalytic activity of these enzymes similarly to that of the receptor-type PTK.

All PTP have a catalytic cysteine residue at the active site [2–4]. Because of this, sulfhydryl oxidizing and alkylating agents are nonreversible inactivators of PTP [5]. In addition, peptides containing non-hydrolyzable phosphotyrosine mimetics such as sulfotyrosine, thiophosphotyrosine, *O*,*O*′-dicarboxymethyltyrosine, phosphonomethylphenylalanine, and 4-[difluoro(phosphono)methyl]phenylalanine have been shown to be inhibitors of PTP [6–13]. In addition to these, the phosphate analogue vanadate inhibits PTP by binding to the active sites of these enzymes [14–16]. It has been suggested that the inhibition of an insulin receptor-specific PTP by vanadate is at least partially responsible for the insulin-mimetic effect of this compound [17, 18]. Although vanadate has been shown to be effective in increasing phosphotyrosine in a variety of

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§ *Abbreviations:* PTK, protein tyrosine kinase(s); DMHV, bis(*N*, *N*-dimethylhydroxamido)hydroxooxovanadate; PTP, protein tyrosine phosphatase(s); [^{14}C]2-DOG, 2-deoxy-D-[U-^{14}C]glucose; FDP, 3,6-fluorescein diphosphate; IBMX, isobutylmethyl xanthine; HRP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium; MAP, mitogen-activated protein; LAR, leukocyte antigen-related protein tyrosine phosphatase; and ECL, enhanced chemiluminescence.

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cell types [19–24], it appears to be ineffective in others [25, 26]. Recently a complex of vanadate with dimethylhydroxylamine, namely DMHV, was synthesized [27]. Like vanadate, this compound inhibits PTP1B and LAR with a K_i in the micromolar range, and the inhibition is reversible upon dilution [28]. DMHV does not carry a charge under physiological conditions, and having four methyl groups makes it quite hydrophobic. Because of this, it is anticipated that it will permeate the cell membrane more easily than vanadate.

In this report we have compared the efficacy of DMHV in the inhibition of PTP in intact cells with the corresponding ability of vanadate. This was probed by measuring the phosphotyrosine levels on various proteins in cell lysates following exposure to these compounds. It is shown here that DMHV was more effective than vanadate in increasing both the phosphotyrosine levels of a variety of proteins in 3T3L1 fibroblasts and the phosphorylation level of the insulin receptor in CHO cells overexpressing the human insulin receptor. The increased effectiveness of DMHV was reflected by the 10- to 20-fold enhancement in the potency of DMHV over vanadate in increasing glucose transport and glycogen synthesis in 3T3L1 adipocytes. It also was demonstrated that, in contrast to vanadate, which was ineffective in increasing phosphotyrosine levels in Jurkat cells, DMHV was quite effective in increasing phosphotyrosine levels in this cell line.

MATERIALS AND METHODS

Materials

3T3L1 cells and Jurkat (E6–1) cells were purchased from the American Type Culture Collection. CHO cells overexpressing the human insulin receptor were a gift from Dr. Donald Steiner [29]. DMEM, penicillin–streptomycin, and newborn-calf and fetal bovine sera were purchased from Life Technologies. HEPES, IBMX, dexamethasone, BSA fraction V, D-(+)glucose, and glycogen were purchased from the Sigma Chemical Co. Human insulin–zinc was obtained from Calbiochem, and vanadium(V) oxide from the Aldrich Chemical Co. DMHV was synthesized as previously described [27]. Cytostar-T 96-well plates, [^{14}C]2-DOG (305 mCi/mmol, 200 μCi/mL), D-[U-^{14}C]glucose (305 mCi/mmol, 200 μCi/mL), and an ECL kit were obtained from Amersham Life Science. Phosphotyrosine antibody 4G10 conjugated to HRP was purchased from Upstate Biotechnology, and MAP kinase antibodies from New England Biolabs. Microscint 20 was obtained from Canberra Packard; 96-well multi-screen GF/C filter plates were obtained from Millipore.

Cell Culture

3T3L1 fibroblasts were seeded in a 96-well Cytostar-T plate (glucose uptake assay) or a Nunc plate (glucose incorporation into glycogen assay), at a density of 4×10^3 cells in 200 μL of medium per well, grown to confluence in regular DMEM containing 25 mM glucose, 2 mM glutamine, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 10% newborn-calf serum, and maintained at confluence for 3–4 days. Cells were differentiated to an adipocyte phenotype using the 6-day protocol of Shepherd *et al.* [30]. Briefly, cells were cultured in DMEM containing 10% fetal bovine serum, 10 mM HEPES, 5 μg/mL of insulin, 0.5 mM IBMX, and 0.25 μM dexamethasone for 2 days, after which IBMX and dexamethasone were removed. Cells were incubated for a further 2 days in DMEM/fetal bovine serum/HEPES/insulin and then subsequently in DMEM/fetal bovine serum. Adipocytes were used within 7–10 days after differentiation was induced. Prior to assay, adipocytes were serum-starved overnight in DMEM containing 0.5% BSA.

Glucose Transport

The transport of [^{14}C]2-DOG was determined using a method outlined in the Proximity News from Amersham Life Science ("The Use of Cytostar-T Scintillating Microplates to Monitor Insulin-Dependent Glucose Uptake by 3T3L1 Adipocytes," May 1996). Briefly, after overnight serum-free incubation, adipocytes were washed with 200 μL of glc buffer (50 mM HEPES, pH 7.4, 120 mM NaCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$, 4.8 mM KCl, and 0.1% BSA). Then cells were depleted of glucose by incubation for 2–3 hr in 200 μL of glc buffer, after which 150 μL of the buffer was removed, 10 μL of each compound diluted in glc buffer was added to the appropriate wells, and the plate was incubated for 45 min at 37°. Then, 10 μL of insulin diluted in glc buffer was added to the appropriate wells, and the plate was incubated for a further 15 min at 37°. Subsequently, the incubation medium was removed, and the cells were washed once, followed by the addition of 50 μL of glc buffer containing 0.1 μCi of [^{14}C]2-DOG. The uptake of [^{14}C]2-DOG was monitored using a Wallac MicroBeta plate counter.

Glycogen Synthesis

After overnight serum-free incubation, the adipocytes were depleted of glucose as described above, and 150 μL of glc buffer was removed. Ten microliters of compound in glc buffer was added to the appropriate wells, and the plate was incubated for 60 min at 37°. To stimulate the cells with insulin, 10 μL of diluted insulin in glc buffer was added, after which 10 μL of 7 mM D-glucose containing 1.0 μCi of D-[U-^{14}C]glucose was added to all the wells, and the plate was incubated for 2 hr at 37°. The final assay volume was 70 μL and contained 1 mM D-glucose. Subsequently, the reaction medium was removed by aspiration, and the cells were washed with 100 μL of ice-cold PBS. Then the cells were disrupted by incubation in 50 μL of 1 N NaOH for 10 min at 60°. The cell homogenates were cooled to ambient temperature and transferred to 96-well GF/C filter plates containing 100 μL of ice-cold ethanol and 2.5 mg/mL of glycogen. The plate was incubated for 2–3 hr at 4°. The

precipitate was filtered under vacuum and washed three times with 250 μL of ice-cold 66% ethanol. The filters were dried at 50° for 1 hr, 50 μL of Microscint 20 was added, and the wells were sealed. The D-[U-^{14}C]glucose incorporated into glycogen was quantitated using a TopCount 96-well liquid scintillation counter.

Treatment of Cells and Preparation of Lysates

3T3L1 cells were cultured in T-25 flasks in DMEM containing 10% fetal bovine serum. CHO cells overexpressing the human insulin receptor were grown in T-25 flasks in α-MEM containing 10% dialysed fetal bovine serum and serum starved overnight before use. The cells were incubated with appropriate compounds in serum-free DMEM at 37°. The medium was aspirated, and the cells were lysed in 250 μL of lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM vanadate, 5 mM H_2O_2, and 1 protease inhibitor mixture tablet (Boehringer Mannheim) per 10 mL of buffer]. The cells were scraped to one corner, incubated on ice for 20 min, and then transferred to microcentrifuge tubes. Jurkat cells were cultured in RPMI 1640 containing 10% fetal bovine serum. The cells were incubated with appropriate compounds in serum-free DMEM at a density of 5×10^6 cells/mL in 50-mL centrifuge tubes for the indicated lengths of times at 37°. One milliliter of cell suspension was harvested and resuspended in 200 μL of the above lysis buffer, vortexed, and incubated on ice for 30 min. All the cell lysates were centrifuged at 14,000 *g* for 10 min at 4°, and the supernatant was used for SDS–PAGE and immunoblotting.

Analysis of Tyrosine Phosphorylation

Cell lysates were electrophoresed using either 4–12% SDS–PAGE (NuPAGE gels) for phosphotyrosine-containing proteins or 10% SDS–PAGE for MAP kinase and blotted onto nitrocellulose membranes as per the manufacturer's suggested protocol. All membranes were blocked in 5% nonfat milk in TBST (Tris–HCl, pH 7.2, 150 mM NaCl, and 0.5% Tween-20) for a minimum of 1 hr at room temperature before being probed. Tyrosine-phosphorylated proteins were detected using a 1:1000 dilution of 4G10 monoclonal antibody conjugated to HRP, followed by ECL. Phosphorylated and nonphosphorylated forms of MAP kinases were detected using a kit from New England Biolabs. Total MAP kinase was detected using a 1:1000 dilution of rabbit polyclonal anti-MAP kinase antibody followed by a 1:2000 dilution of HRP-conjugated anti-rabbit antibody. The blot was stripped (60° for 1 hr in 10% SDS, 150 mM 2-mercaptoethanol, and 62.5 mM Tris–HCl, pH 6.8), washed in TBST, blocked, and reprobed with a 1:1000 dilution of rabbit polyclonal anti-phosphorylated MAP kinase antibody followed by a 1:2000 dilution of HRP-conjugated anti-rabbit antibody. ECL was performed on all probed blots.

Reversibility of Inhibition of PTP by DMHV in Jurkat Cells

Cells were treated with appropriate compounds as described above. Then the cells were transferred to a 1.5-mL tube and centrifuged briefly, and the pellets were resuspended in 200 μL of buffer [20 mM Tris–HCl, pH 7.5, 5 mM dithiothreitol, 2 mM EDTA, and 1 protease inhibitor mixture tablet (Boehringer Mannheim) per 10 mL]. The cells underwent three cycles of freezing in liquid N_2 and thawing in a 37° water bath. The lysate was centrifuged at 14,000 *g* for 10 min at 4°. The pellet was resuspended in 200 μL of the above buffer containing 0.5% Triton X-100. The membrane proteins were solubilized for 30 min at 4° with constant inversion on a rotor. The samples were centrifuged as above, and the supernatant was assayed for phosphatase activity by measurement of FDP hydrolysis [16]. Ten microliters of sample was added to 180 μL of buffer [50 mM bis-Tris, pH 6.2, 2 mM EDTA, and 5 mM *N*,*N*′-dimethyl-*N*,*N*′-bis(mercaptoacetyl)hydrazine]. The reaction was initiated by the addition of FDP to a final concentration of 20 μM. The fluorescence of the product fluorescein monophosphate [16] was measured at room temperature in a CytoFluor II fluorescence plate reader (PerSeptive) at an excitation wavelength of 440 nm (slit width 20 nm) and an emission wavelength of 530 nm (slit width 25 nm). The formation of the product fluorescein monophosphate increased linearly with time as well as protein concentration. The rate of FDP hydrolysis was normalized to the protein concentration, as determined by the Bradford assay.

RESULTS

Inhibition of Protein Tyrosine Phosphatases in 3T3L1 Fibroblasts and CHO Cells Overexpressing the Insulin Receptor

DMHV is a potent and reversible inhibitor of PTP with a potency slightly lower than vanadate [28]. DMHV is an uncharged molecule at physiological pH; consequently, it was anticipated that it would permeate the cell membrane more easily than does vanadate. This may influence the efficacy of DMHV as a PTP inhibitor in intact cells. The inhibition of PTP in intact cells can be ascertained and quantitated by measuring the phosphotyrosine levels of various proteins, provided the relevant PTK are active. To address this issue, 3T3L1 fibroblasts were incubated with 1 mM concentrations of either vanadate or DMHV for 0–120 min, and whole cell lysates were prepared. They were separated by SDS–PAGE, transferred to nitrocellulose, and probed for phosphotyrosine using 4G10 monoclonal antibody conjugated to HRP. Vanadate was effective in increasing phosphotyrosine levels of several proteins, most prominently those proteins with molecular masses of 105, 150, and 200 kDa (Fig. 1A). From the time–course data, the phosphotyrosine levels started to increase at about 30 min and were maximal by 120 min. In the case of DMHV-treated cell lysates, an essentially identical profile was

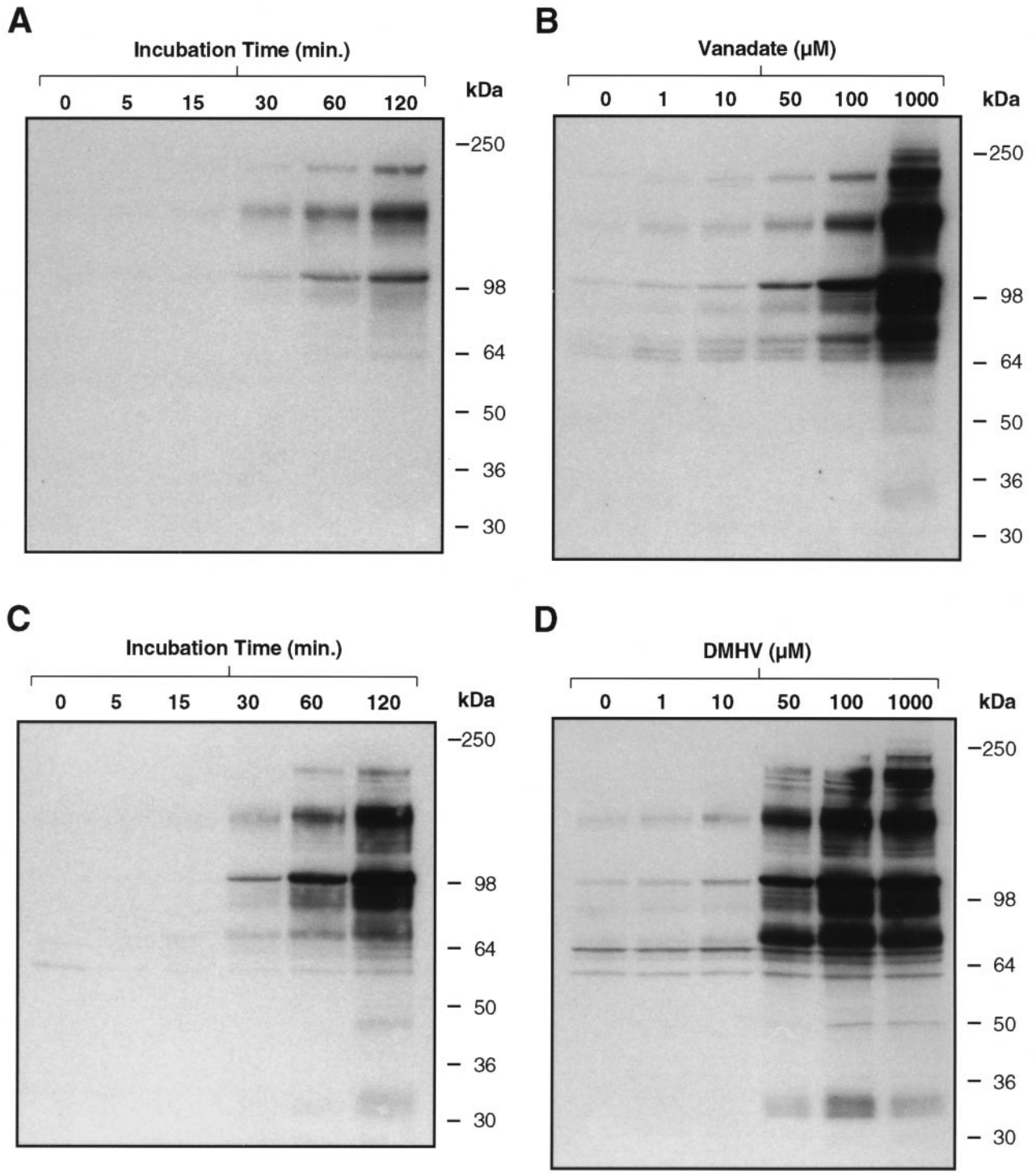

FIG. 1. Comparison of the effect of vanadate and DMHV in increasing phosphotyrosine levels in 3T3L1 fibroblasts. 3T3L1 fibroblasts were incubated with 1 mM concentrations of either vanadate (A) or DMHV (C) for the times indicated in the figure or with various concentrations of either vanadate (B) or DMHV (D) as indicated for 2 hr before preparing lysates and probing for phosphotyrosine levels as described in Materials and Methods. The ECL exposure times for panels A and C were 60 sec, and those for panels B and D were 120 sec. Data from a typical experiment (out of four independent experiments) are presented.

observed (Fig. 1C); however, the intensity of the phosphotyrosine bands was higher than that observed with vanadate. The concentration–response relationship for vanadate (Fig. 1B) and DMHV (Fig. 1D) suggested that vanadate is less potent than DMHV in increasing phosphotyrosine levels. When the cells were incubated with peroxovanadate, an

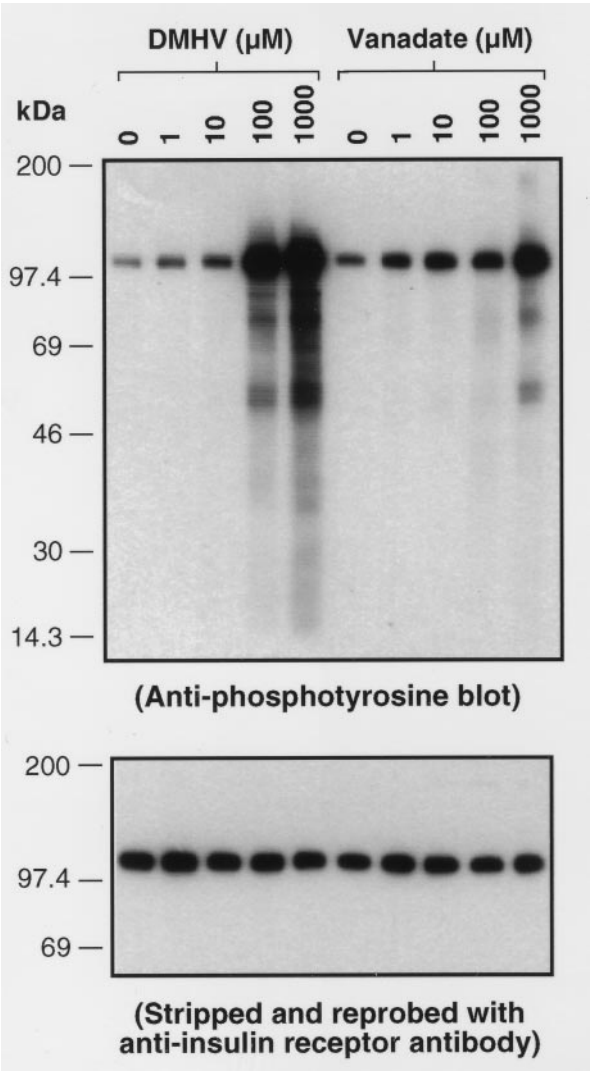

FIG. 2. Comparison of the effects of vanadate and DMHV in increasing the phosphorylation level of the insulin receptor. CHO cells overexpressing the human insulin receptor were incubated with various concentrations of either vanadate or DMHV for 50 min. The cells were lysed and probed for phosphotyrosine (top blot) as described in Materials and Methods. The blot was stripped (60° for 1 hr in 10% SDS, 150 mM 2-mercaptoethanol, and 62.5 mM Tris–HCl, pH 6.8), washed in TBST, blocked, and reprobed with an anti-insulin-receptor antibody (bottom blot). Data from a representative experiment (out of two experiments) are presented.

irreversible inactivator of PTP [16], a rapid increase in phosphotyrosine levels of the same proteins as those observed with vanadate or DMHV treatment was seen (data not shown). The increase in phosphotyrosine levels was evident as early as 1 min after treatment with peroxovanadate, became maximal within 5–10 min, and remained maximal for up to 60 min (data not shown).

The data in Fig. 2 provide a comparison of the concentration–response of both the vanadate- and the DMHV-induced increase in the phosphotyrosine levels in CHO cells overexpressing the human insulin receptor. In this cell line, the major protein that undergoes a concentration-dependent increase in phosphotyrosine content has a molecular mass of 98 kDa and is the β subunit of the insulin receptor. DMHV was more effective than vanadate in increasing the phosphotyrosine level of the β subunit of the insulin receptor. The amount of insulin receptor in the lysates, as anticipated, was unaffected by treatment with vanadate or DMHV (Fig. 2, lower panel).

Enhancement of Glucose Transport and Glycogen Synthesis by Insulin and PTP Inhibitors

To ascertain some of the biological consequences of the above effects, namely increase in insulin-receptor phosphorylation, the ability of vanadate and DMHV to mimic insulin was assessed. An insulin-sensitive cell line, 3T3L1 adipocytes, was chosen for this study. 3T3L1 adipocytes were treated with various concentrations of insulin, vanadate, or DMHV, and the transport of glucose was measured continuously by measuring the uptake of ^{14}C-labeled 2-deoxyglucose using Cytostar plates (Fig. 3 A–C). Insulin caused a concentration-dependent increase in glucose transport of about 160% (Fig. 3A) with an EC_{50} of 23 ± 7 nM (Table 1). Vanadate caused a 140% increase in glucose transport (Fig. 3B) with an EC_{50} of 243 ± 67 μM (Table 1), whereas DMHV caused a similar increase in glucose transport with an EC_{50} of 27 ± 3 μM (Fig. 3C and Table 1).

A second bona fide and biologically relevant effect of insulin is to increase the synthesis of glycogen by activation of the rate-limiting enzyme glycogen synthase [31]. Hence, we compared the efficacy of insulin with that of both vanadate and DMHV to increase glycogen synthesis in 3T3L1 adipocytes by measuring the incorporation of ^{14}C-labeled glucose into glycogen (Fig. 3, D–F). Insulin caused a concentration-dependent increase in glycogen synthesis (Fig. 3D). The maximal effect of insulin was about 5-fold, with an EC_{50} of 0.9 ± 0.2 nM (Table 1). Similarly, vanadate caused a concentration-dependent increase in glycogen synthesis of about 3- to 4-fold with an EC_{50} of 481 ± 66 μM (Fig. 3E and Table 1). DMHV also caused a concentration-dependent increase in glycogen synthesis of similar magnitude with an EC_{50} of 22 ± 4 μM (Fig. 3F and Table 1). From the above experiments on glucose transport and glycogen synthesis, it is quite clear that DMHV is about 10- to 20-fold more potent than vanadate (Table 1). In contrast, with purified PTP, DMHV is a 2- to 3-fold less effective inhibitor than is vanadate [28].

Inhibition of PTP in Jurkat Cells

It is known that vanadate is ineffective as a PTP inhibitor in Jurkat cells [26]. Hence, it was of interest to examine the effect of DMHV as a PTP inhibitor in this cell line. DMHV, in contrast to vanadate, caused a time-dependent increase in the phosphotyrosine level of several proteins in Jurkat cells (Fig. 4A). Most prominent among these were proteins with molecular masses of 150, 130, 97, 70, 64, 55, and 40 kDa. The effect of DMHV was evident as early as 15

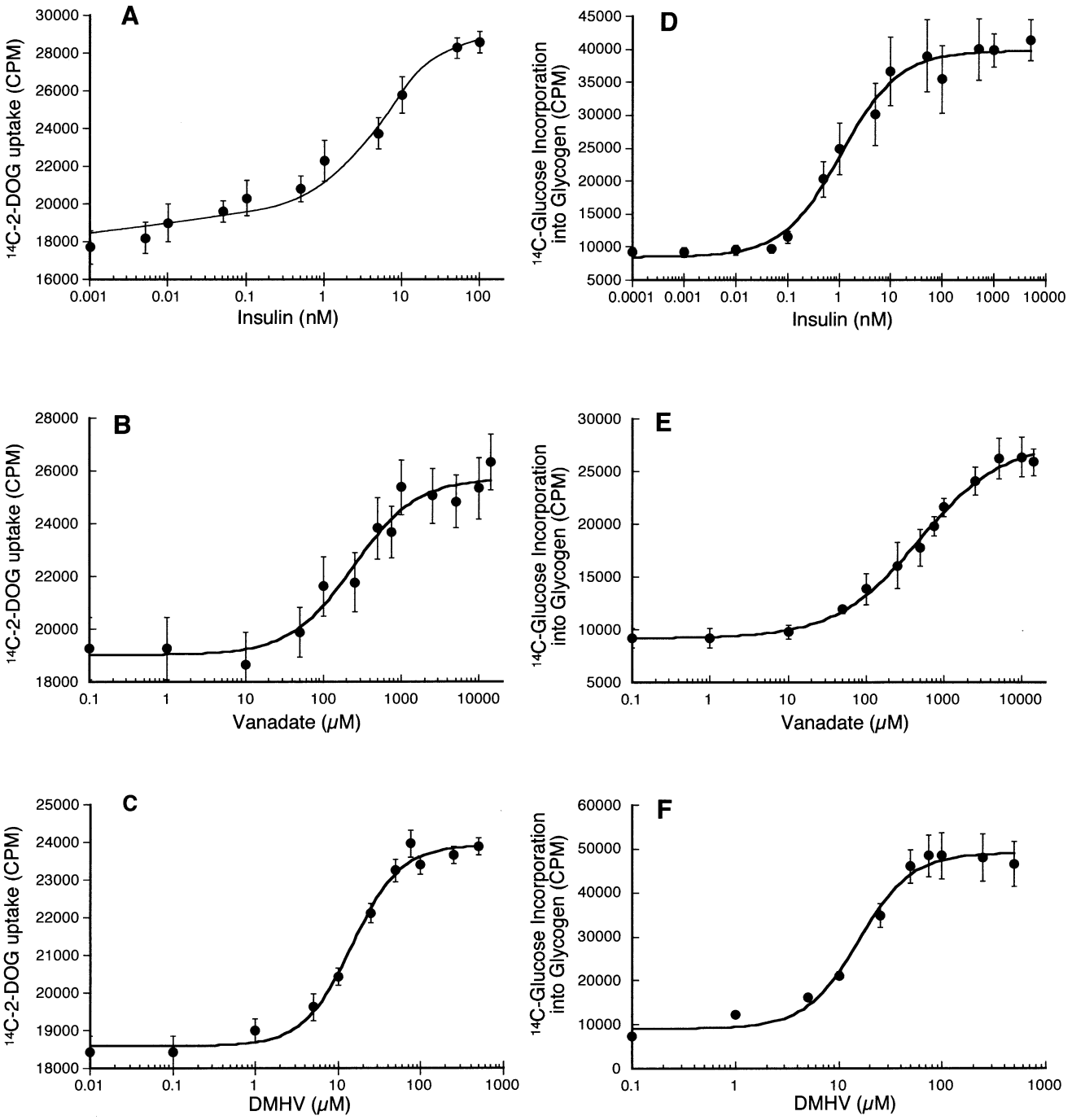

FIG. 3. Stimulation of [^{14}C]2-DOG uptake and activation of glycogen synthesis in 3T3L1 adipocytes by insulin, vanadate, and DMHV. Cells were incubated with insulin for 15 min (A) or with vanadate (B) or DMHV (C) for 1 hr prior to addition of 0.1 μCi of 2-deoxy-D-[U-^{14}C]glucose to initiate glucose uptake as described in Materials and Methods. For glycogen synthesis measurement, cells were preincubated for 1 hr in reaction medium containing increasing concentrations of vanadate (E) and DMHV (F). This was followed by the addition of increasing concentrations of insulin (D) to the appropriate wells and by the addition of D-glucose to all the wells. The final D-glucose concentration was 1 mM, containing 1.0 μCi of D-[U-^{14}C]glucose. The plate was incubated for 2 hr at 37°. The incorporation of radiolabeled glucose into glycogen then was quantitated as described in Materials and Methods. The results for both glucose uptake and glycogen synthesis are the means ± SEM of four determinations.

min. We also examined the phosphorylation status of MAP kinases in response to DMHV treatment. Data in Fig. 4B show that both p42 and p44 isoforms of MAP kinase were phosphorylated in response to DMHV. The mechanism of inhibition of PTP by DMHV in Jurkat cells was investigated by measuring the FDP hydrolysis activity of the crude Jurkat cell membranes. The major membrane-associated PTP in Jurkat cell membrane is CD45, and this accounts for

TABLE 1. EC_{50} Values for insulin, vanadate, and DMHV, for the stimulation of 2-DOG transport and glycogen synthesis in 3T3L1 adipocytes

Assay	Insulin (nM)	Vanadate (μM)	DMHV (μM)
[^{14}C]-2-DOG uptake	23 ± 7 (n = 24)	243 ± 67 (n = 4)	27 ± 3 (n = 16)
[^{14}C]-Glucose incorporation into glycogen	0.9 ± 0.2 (n = 28)	481 ± 66 (n = 4)	22 ± 4 (n = 12)

The EC_{50} values (mean ± SD) for glucose transport and glycogen synthesis in adipocytes were determined as in Fig. 3.

about 75% of the PTP activity.* The PTP activity of membranes prepared from Jurkat cells pretreated with DMHV was no different from the control membranes. This suggested that the inhibition of PTP by DMHV occurred in a reversible manner (Fig. 5), similar to what has been observed with purified PTP. This result is in contrast to the observations with peroxovanadates, which irreversibly inactivate purified PTP [16] and PTP in intact cells (Fig. 5).

DISCUSSION

Vanadate has a wide variety of effects in biological systems [32]. Of these influences, the insulin-mimetic property of vanadate has attracted the greatest attention [17, 18]. Vanadate has been shown to normalize blood glucose levels in streptozotocin-induced diabetic rats, correct hyperglycemia, and improve glucose tolerance in both *ob/ob* and *db/db* mice and in *fa/fa* rats [33–37]. Recent clinical trials in humans have shown enough improvement in insulin sensitivity to consider vanadate as potentially useful for treating insulin-dependent and non-insulin-dependent diabetes mellitus [38, 39]. It has been suggested that this effect of vanadate is due to its ability to inhibit PTP involved in dephosphorylating the insulin receptor, thus resulting in prolongation of insulin signaling [17, 18]. *In vitro*, vanadate has been shown to be a reversible inhibitor of PTP with a micromolar affinity [14–16]. This may be because of the ability of vanadate to adopt a trigonal bipyramidal structure mimicking the transition state of phosphoryl transfer reactions [40]. Compared with vanadate, peroxovanadates are much more potent and irreversible inactivators of PTP [16]. A variety of vanadate complexes including bismaltooxovanadates, peroxovanadates with different ancillary ligands, have been synthesized and studied in an attempt to come up with more selective and cellularly active, but less toxic insulin-mimetic agents [41, 42].

Recently, we synthesized DMHV and showed that it inhibited the enzymes PTP1B and LAR with a K_i of 1–2 μM, only 2- to 3-fold higher than that of vanadate [28]. The inhibition by DMHV, like that of vanadate but in contrast to that of peroxovanadates, was reversible upon dilution. DMHV is uncharged at physiological pH ranges and consequently might enter cells more freely than vanadate. Because of this, we were interested in exploring how well it inhibited PTP in intact cells. The quantitation of the phosphotyrosine levels of proteins in the cell lysate in response to a compound is a measure of inhibition of PTP in intact cells, provided the relevant tyrosine kinases are active. Vanadates appear to have no effect on PTK [22, 43]. Therefore, any increase in the phosphotyrosine level on a protein in response to the presence of vanadate suggests that the PTP acting on that protein was inhibited, and it is this that is responsible for the observed enhancement of phosphotyrosine levels. The phosphotyrosine level of several proteins in 3T3L1 fibroblasts and of the insulin receptor in CHO cells overexpressing the insulin receptor was enhanced in response to vanadate and DMHV (Figs. 1 and 2). In both cell lines, DMHV was slightly more potent. In 3T3L1 adipocytes, a classical insulin-sensitive cell line,

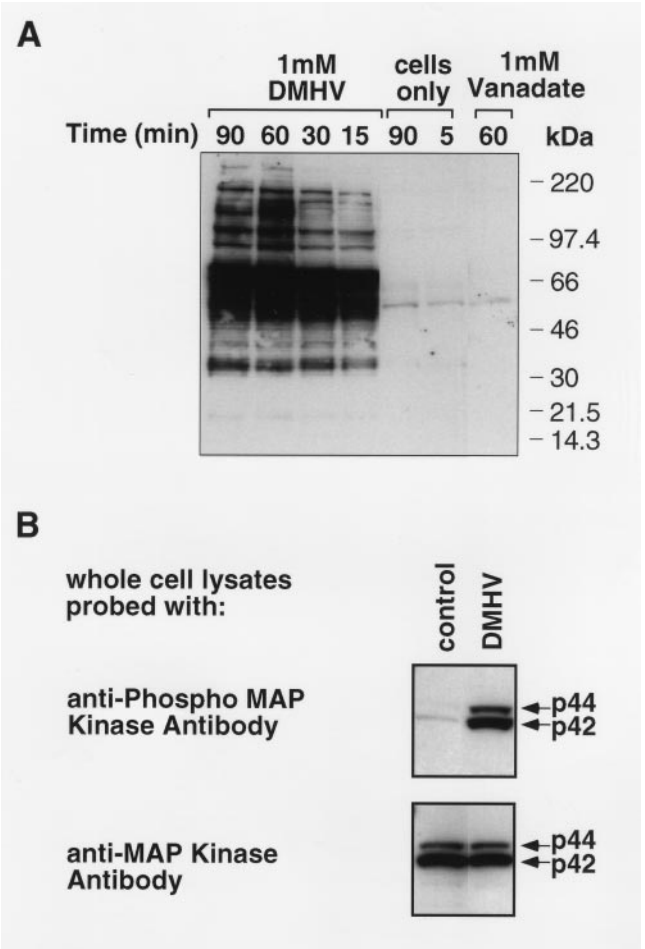

FIG. 4. Effect of DMHV on phosphotyrosine levels in Jurkat cells. Jurkat cells were incubated with 1 mM DMHV or vanadate (A) for varying lengths of time before lysing and probing for phosphotyrosine as described in Materials and Methods. In panel B, the phosphorylation status of MAP kinases p42 and p44 was determined as described in Materials and Methods, in an aliquot of lysate prepared from cells exposed to 0 (control) or 1 mM DMHV for 60 min. Data from a typical representative experiment (out of three experiments) are presented.

* Q. Wang, unpublished observation. Cited with permission.

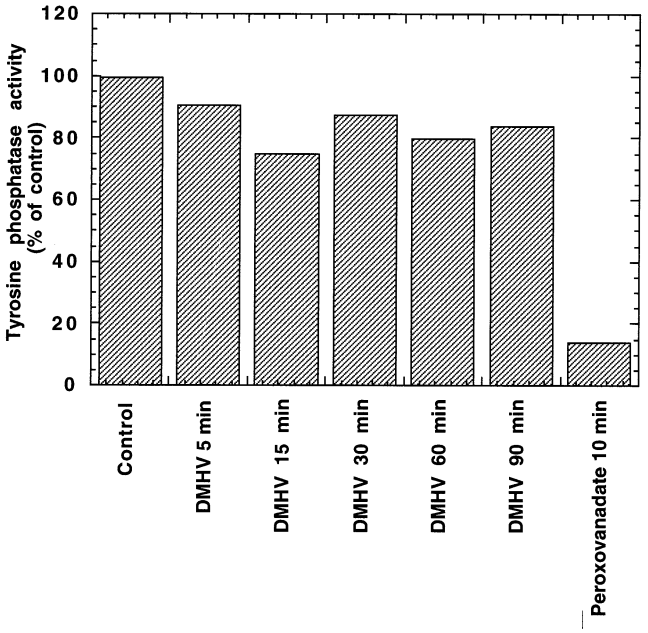

FIG. 5. Reversibility of the inhibition of tyrosine phosphatases by DMHV in Jurkat cells. Jurkat cells were incubated with 1 mM DMHV (5–90 min) or 100 μM peroxovanadate for 10 min before lysing and preparing membranes. The phosphatase activities of the solubilized membranes were measured as described in Materials and Methods. The specific activity of the PTP from solubilized membranes from control cells was 6.6 pmol of fluorescein monophosphate formed/min/μg of protein and was taken as 100% activity. The average data from a representative experiment (carried out in triplicate) are presented.

DMHV stimulated glucose transport (Fig. 3 A–C) and glycogen synthesis (Fig. 3 D–F) as effectively as insulin. For both of these functionalities, DMHV was 10- to 20-fold more potent than vanadate.

The studies described thus far indicated that the only difference between vanadate and DMHV was in terms of potency. A surprising and exciting observation was made when we examined the effect of DMHV in Jurkat cells. In contrast to vanadate, which is ineffective as a PTP inhibitor in this cell line [26], DMHV was a potent inhibitor of PTP activity. This was reflected by the appearance of several phosphotyrosine-containing proteins as early as 15 min after treatment (Fig. 4A). As a specific example, we investigated the phosphorylation status of MAP kinases, whose phosphorylation levels also were enhanced by DMHV (Fig. 4B). The mechanism of inhibition of PTP by DMHV was investigated by measuring the PTP activity of Jurkat cell membranes (CD45 is the major membrane-associated PTP and accounted for about 75% of the PTP activity measured under our experimental conditions). Similar to the observation with purified PTP, the inhibition of PTP by DMHV in intact Jurkat cells was reversible (Fig. 5). This contrasts with the irreversible inactivation of PTP by peroxovanadates.

The increased cellular potency of DMHV over vanadate may derive from its hydrophobicity and from its uncharged state. These properties could well enhance the ability of DMHV to cross the cell membrane. It is also likely that the ability of DMHV to form complexes with various constituents of the cell is different from that of vanadate [44]. Consequently, more of the compound may be available for inhibition of PTP, thus accounting for the enhanced potency of DMHV over vanadate in intact cells. The successful demonstration of enhanced insulin sensitivity in the clinical trials with vanadate, combined with the possibility to moderate the potency of vanadate with appropriate ligands, suggests that it will be possible to synthesize selective PTP inhibitors. Such compounds will be useful in deciphering the signal transduction pathways modulated by specific PTP. In addition, they have the potential to be useful for modulating the function of known therapeutic targets as well as those yet to be discovered.

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