0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 6
Printed in U.S.A.

Antilipolytic Actions of Vanadate and Insulin in Rat Adipocytes Mediated by Distinctly Different Mechanisms*

JINPING LI, GERARD ELBERG†, NATESAMPILLAI SEKAR, ZHI BIN HE, AND YORAM SHECHTER‡

Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT

Vanadate, which mimics the biological effects of insulin, also inhibits lipolysis in rat adipocytes. Here we demonstrate that the antilipolytic effect of vanadate differs from that of insulin at least by the five following criteria: 1) vanadate inhibits lipolysis mediated by high (supraphysiological) concentrations of catecholamines; 2) vanadate antagonizes $(Bu)_2$cAMP-mediated lipolysis; 3) vanadate antagonizes isobutylmethylxanthine-dependent lipolysis, 4) vanadate inhibits lipolysis mediated by okadaic acid; and 5) wortmannin, which blocks the antilipolytic effect of insulin, fails to block vanadate-mediated antilipolysis. Vanadate does activate phosphoinositol 3-kinase, and wortmannin blocks this activation.

Our working hypothesis assumes that all of the insulin-like effects of vanadate, including antilipolysis, are initiated by the inhibition of protein phosphotyrosine phosphatases (PTPases). Among documented PTPase inhibitors we found that $VOSO_4$ (oxidation state +4), several organic vanadyl compounds (+4), zinc (Zn^{2+}), tungstate (W), and molybdate (Mo) also had antilipolytic activity. The order of potency was vanadyl acetylacetonate $\geq$ $VOSO_4$ $\geq$ $NaVO_3$ $\geq$ vanadyldipicolinate $>$ Zn^{2+} » W $>$ Mo, and it correlated better with the inhibition of adipose membranal-PTPases in cell-free experiments. We have concluded that the antilipolytic effect of vanadate is 1) mechanistically distinct from that of insulin, 2) independent of phosphoinositol 3-kinase activation, and 3) independent of the lipolytic cascade. We also strongly suggest that the antilipolytic effect of vanadate emanates from inhibiting adipose membranal, rather than cytosolic PTPases, and present preliminary data showing distinct differences in catalysis between these two PTPase categories. Overall, the study indicates that antilipolysis can be manifested via alternative, insulin-independent, signal-transducing pathways. (*Endocrinology* **138:** 2274–2279, 1997)

INSULIN IS perhaps the sole anabolic hormone that also inhibits catabolic processes. In adipose cells, insulin antagonizes lipolysis mediated by physiological concentrations of lipolytic hormones (reviewed in Ref. 1). These hormones (*i.e.* catecholamines, ACTH, and glucagon) share the ability to activate adenylate cyclase and increase intracellular cAMP. cAMP-dependent protein kinase is then activated, followed by phosphorylation and activation of hormone-sensitive triglyceride lipase (HSL) (2, 3). Translocation of HSL from the cytosol to the lipid storage droplet may play a role in lipolysis as well (4).

Little is known about the mechanism(s) by which insulin antagonizes lipolysis. Several sites along the lipolytic cascade are potentially sensitive to insulin: adenylate cyclase may be inhibited (5–7), low K_m cAMP phosphodiesterase (8–11) may be activated, protein phosphoserine phosphatase 2A, which dephosphorylates HSL at site 2 may be stimulated (12, 13). Many studies either support or disprove an insulin effect at any of the above-mentioned sites. The possibility that the hormone acts in concert at several points along this cascade is certainly feasible. In intact adipocytes, lipolysis induced by high (supraphysiological) concentrations of catecholamines (14), by the presence of the cAMP phosphodiesterase inhibitor, isobutylmethylxanthine (15), by $(Bu)_2$cAMP (15), or by okadaic acid (16) cannot be antagonized by insulin. Okadaic acid is a powerful inhibitor of protein phosphoserine phosphatose 2A (IC_{50} = 0.2 nM), which is possibly the principal enzyme that dephosphorylates HSL (17).

Vanadate mimics virtually all the biological effects of insulin, including inhibition of lipolysis mediated by physiological concentrations of isoproterenol (18, 19). As with insulin, the mechanism(s) by which vanadate antagonizes lipolysis is still unknown. Recent studies from our laboratory, however, have elucidated some of the basic mechanisms by which vanadate exerts its insulin-like effects on glucose and fat metabolism in rat adipocytes. We observed that quercetin, which inhibits insulin receptor tyrosine kinase (InsRTK)-catalyzed phosphorylation and insulin-mediated effects in intact cells, failed to inhibit the very same effects when triggered with vanadate (20). The assumption that several vanadate effects require endogenous tyrosine phosphorylation led to the discovery of a Co^{2+}-dependent *N*-ethylmaleimide insensitive nonreceptor, cytosolic protein tyrosine kinase (CytPTK) with a molecular mass ~of 53 kDa (21, 22). The enzyme is activated (3- to 5-fold) in vanadate-

Received October 23, 1996.

Address all correspondence and requests for reprints to: Dr. Yoram Shechter, The Weizman Institute of Science, Department of Biochemistry, 76100 Rehovot, Israel.

* This work was supported in part by grants from the Minerva Foundation (Germany), the Rowland Shaefer Contribution to Diabetes Research, the Israel Ministry of Health, and the Israel Academy of Sciences and Humanities.

† Recipient of the Levi Eshkol Post Doctoral Fellowship from the Ministry of Science and Technology in Israel.

‡ Incumbent of the C. H. Hollenberg Chair in Metabolic and Diabetes Research, established by the Friends and Associates of Dr. C. H. Hollenberg of Toronto, Canada.

pretreated adipocytes and is strongly inhibited by staurosporine (IC_{50} = 2 nM) (21, 22). In intact adipocytes, staurosporine blocked two effects of vanadate, lipogenesis and glucose oxidation, but did not influence two other effects, enhancing hexose uptake and inhibiting lipolysis. The ability of vanadate to activate CytPTK was also preserved in a cell-free system and could be mimicked by tungstate, molybdate, and phenylarsine oxide (23), which are documented inhibitors of protein phosphotyrosine phosphatases (PTPases) (24) Thus, CytPTK activation appears to be secondary to PTPase inhibition. Tungstate and molybdate also mimic the actions of insulin in an insulin receptor-independent fashion (25, 26). Our current working hypothesis contends that all of the manifested insulin effects of vanadate are initiated by inhibiting PTPases.

The purpose of this work was to obtain insight into the antilipolytic action of vanadate. Using agents to trigger lipolysis at known locations, our first aim was to map the site(s) affected by vanadate along the lipolytic pathway. If the mechanism of antilipolysis by vanadate proved to be different from that of insulin, then we attempted to determine its pathway, based on the progress made in understanding the activating effects of the metalooxide on glucose metabolism.

Materials and Methods

D-[U-^{14}C]Glucose and 2-D-[1,2-^{3}H]deoxyglucose were purchased from New England Nuclear (Boston, MA), Collagenase type I (134 U/mg) was obtained from Worthington Biochemical Corp. (Freehold, NJ), and okadaic acid and wortmannin were purchased from Sigma Chemical Co. (St. Louis, MO). Porcine insulin was purchased from Eli Lilly Co. (Indianapolis, IN), vanadyl acetylacetonate [$VO(acac)_2$] from Aldrich Chemical Co. (Milwaukee, WI), and anti-phosphotyrosine (PTyr)-conjugated to agarose (1G2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, contained 110 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, and 1.3 mM $MgSO_4$. Vanadyl-dipicolinate, prepared as described previously (27), was donated by Dr. D. C. Crans (Department of Chemistry, Colorado State University, Fort Collins, CO). All chemicals and reagents used in this study were of analytical grade.

Cell preparation and bioassays

Rat adipocytes. Rat adipocytes were prepared from the fat pads of male Wistar rats (150–200 g) by collagenase digestion (28). Cell preparations showed more than 95% viability by trypan blue exclusion at least 3 h after digestion. Lipogenesis (the incorporation of [U-^{14}C]glucose into lipids) was performed essentially according to the method of Moody *et al.* (29). Glucose transport was carried out using 2-D-[1,2-^{3}H]deoxyglucose (30).

Fractionation of adipose-PTPases. Freshly prepared rats adipocytes were homogenized in the presence of various proteolytic inhibitors after centrifugation at 40,000 × *g* and fractionation into cytosolic (supernatant) and Triton-soluble plasma membrane PTPases (26).

Lipolysis. Lipolysis was allowed to proceed on fat cell suspensions (~3 × 10^5 cells/ml) in KRB (pH 7.4)-0.7% BSA, at 37 C for 1 h after stimulation with isoproterenol. Aliquots from the medium were then taken. BSA was removed by trichloroacetic acid precipitation, and the glycerol content in the medium was determined by a spectroscopic assay (Triglyceride C-37 rapid/stat test, Pierce Chemical Co., Rockford, IL) (16).

Adipose PTPase activity. Adipose PTPase activity was evaluated using ^{32}P-labeled poly-Glu_4Tyr as the substrate (31). Poly-Glu_4Tyr was phosphorylated with enriched (wheat-germ agglutinin-agarose purified) insulin receptor from rat liver and [γ-^{32}P]ATP as described in detail previously (32). The assay was performed for 8 min at 37 C in a 60-μl final volume of 25 mM imidazole-HCl buffer, pH 7.2, containing 1 mg/ml [^{32}P]poly-Glu_4Tyr and about 2 μg from fresh adipocyte extracts (either 40,000 × *g* supernatant fraction or Triton-solubilized plasma membranes; final concentration of Triton X-100 in assay, 0.1%). The reaction was terminated by spotting aliquots (50 μl) onto Whatman 3 MM filter paper, followed by several washings with 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. The paper strips were dried and analyzed in a β-spectrometer. The amount of ^{32}P hydrolysis from [^{32}P]poly-Glu_4Tyr was then determined.

Phosphoinositol (PI) 3-kinase activity. PI3-kinase activity was measured essentially as described previously (33, 34) with slight modifications. Suspensions of adipocytes (2×10^6 cells) were incubated in the presence and absence of insulin or vanadate for 20 min at 37 C. The cells were separated from the medium and lysed in chilled buffer containing 20 mM Tris-HCl (pH 7.4), 145 mM NaCl, 10% glycerol, 5 mM EDTA, 0.2 mM $NaVO_3$, 0.1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The fat was removed, and the homogenate was supplemented with Triton X-100 (final concentration, 1%), incubated for 1 h at 4 C, and then centrifuged at 20,000 × *g* for 30 min. The soluble fraction was incubated with anti-PTyr-agarose-conjugate (1G2) overnight at 4 C. This procedure was reported to clear greater than 90% of the anti-PTyr-immunoprecipitable PI3-kinase activity (33).

The immunoprecipitates were washed and assayed for PI3-kinase. The assay contained in a final volume of 0.1 ml, 40 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.2 mM phosphatidylinositol, 0.2 mM phosphatidylserine, 5 mM $MgCl_2$, and 0.1 mM (1 μCi) [γ-^{32}P]ATP. The reaction was carried out at 30 C for 30 min and stopped by the addition of 50 μl 8% $HClO_4$ and 0.45 ml $CHCl_3$-methanol (1:2). After vigorous stirring, 0.15 ml $CHCl_3$ and 0.15 ml 8% $HClO_4$ were added to the mixture. The organic phase was evaporated to dryness and dissolved in 20 μl $CHCl_3$-methanol (95:5) to be spotted on a silica gel plate (Silica Gel 60, Merck, Rahway, NJ). The plate was developed in $CHCl_3$-CH_3OH-H_2O-25% NH_4OH (60:47:10:3, vol/vol/vol/vol). The dried plate was visualized for the reaction products with a Fuji BAS2000 Bioimaging analyzer.

Protein concentration. The protein concentration was determined by the method of Bradford (35). All assays were performed in either duplicate or triplicate.

Results

Vanadate inhibits lipolysis mediated by high isoproterenol concentrations

Figure 1 shows inhibition of lipolysis by vanadate and insulin at increasing isoproterenol concentrations. Insulin fully inhibited lipolysis when the concentration of isoproterenol in the medium was 0.001–0.01 μM. At higher isoproterenol concentrations, the capacity of insulin to inhibit lipolysis was reversed. Thus, at 0.1, 1.0, and 10 μM isoproterenol, the extent of inhibition amounted to 56 ± 4%, 12 ± 2%, and 4 ± 0.3% only (Fig. 1). Higher concentrations of insulin (*i.e.* 30 nM) also could not reverse lipolysis obtained at higher isoproterenol concentrations. In contrast, vanadate (0.3 mM) fully inhibited lipolysis mediated by all concentrations of isoproterenol. Basal lipolysis, obtained in the absence of isoproterenol, was not affected by vanadate (not shown).

Vanadate inhibits lipolysis mediated by nonhormonal activators of lipolysis

Vanadate completely antagonized lipolysis triggered by cholera toxin and forskolin, activators of adenylate cyclase downstream of the catecholamine receptor (36, 37), and by $(Bu)_2$cAMP, which activates protein kinase A downstream of adenylate cyclase (not shown). Vanadate also inhibited lipolysis induced by okadaic acid (Fig. 1B), which probably acts at the end of the lipolytic cascade by activating HSL (17, 38). As reported previously, lipolysis induced by each of

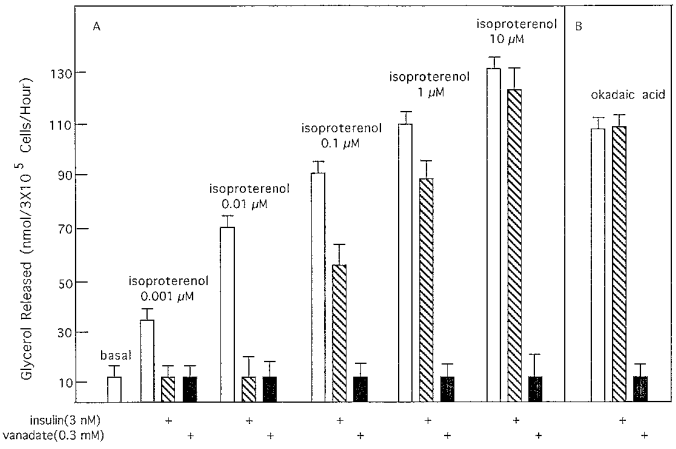

FIG. 1. Inhibition by insulin and vanadate of lipolysis mediated by a wide range of concentrations of isoproterenol and by okadaic acid. Lipolysis was allowed to proceed for 1 h at 37 C using freshly prepared fat cell suspensions (3×10^5 cells/ml) in KRB buffer (pH 7.4)-0.7% BSA in the presence or absence of the indicated concentrations of isoproterenol, insulin (3 nM), or vanadate (0.3 mM). In B, lipolysis was carried out for 3 h using 2 μM okadaic acid as the lipolytic agent and in the presence or absence of insulin (3 nM) or vanadate (0.3 mM). Cells were then removed, BSA was precipitated with trichloroacetic acid, and the glycerol content in the medium was determined (see *Materials and Methods*). The results are the mean ± SEM of four experiments.

these nonhormonal agents is not, with the exception of cholera toxin, antagonized by insulin (14–16).

Role of PI3-kinase

Recent data indicate that insulin activates PI3-kinase (33, 39, 40). Wortmannin, a selective and potent inhibitor of this enzyme, antagonizes all of the metabolic effects of the hormone, including inhibition of lipolysis in rat adipocytes (33). Like insulin, vanadate also stimulates PI3-kinase in rat adipocytes (Fig. 2A). Fifty percent activation is evident at 70–100 μM vanadate (Fig. 2B), which is within the same concentration range as its insulin-like effects on glucose and fat metabolism in this cell type (41). At high vanadium concentrations (1 mM), the extent of PI3-kinase activation exceeded that of insulin. As with insulin, wortmannin blocked the activating effects of vanadate on hexose uptake and lipogenesis (Fig. 3, A and B). Inhibition was evident at very low concentrations of wortmannin, and with similar efficacy (IC_{50} = 4–5 nM; Fig. 3B). However, whereas this fungal metabolite antagonized the antilipolytic effect of insulin as well (33), it did not reverse the antilipolytic effect of vanadate (Fig. 4). Wortmannin could not reverse the antilipolytic effect of vanadate even when the latter was applied at lower doses (*i.e.* 20–50 μM; not shown).

Antilipolytic potencies of vanadate and other documented PTPase inhibitors

In addition to vanadate (oxidation state +5), vanadyl sulfate ($VOSO_4$; oxidation state +4), several organic vanadyl compounds, zinc (Zn^{2+}), tungstate (WO_4^{-3}), and molybdate (MoO_4^{-2}) were also antilipolytic. The median dose (ED_{50}) values are summarized in Table 1 (column A). Vanadyl and vanadyl(acac)$_2$ were about twice as potent as vanadate in

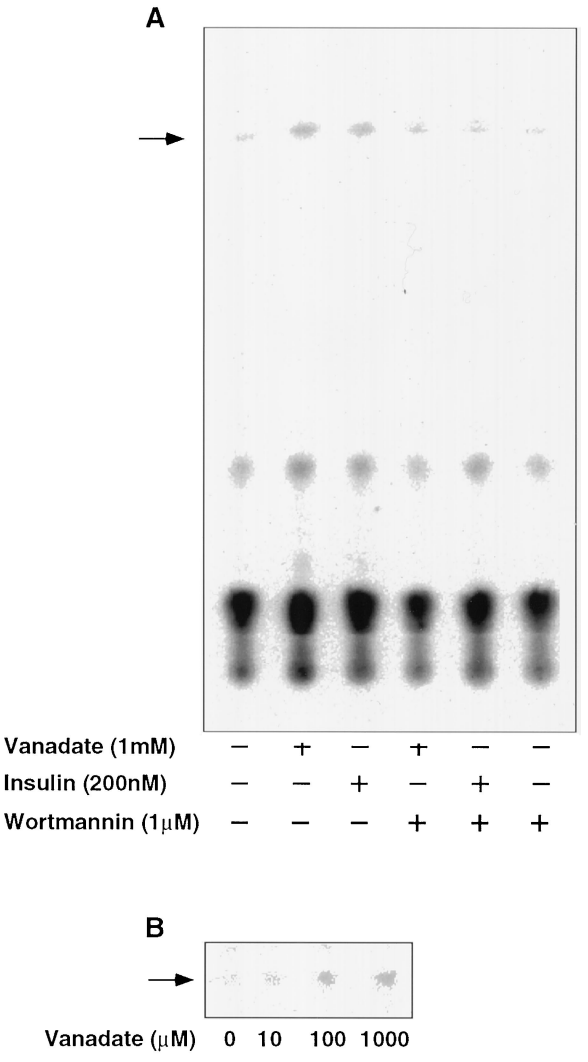

FIG. 2. Activation of adipose PI3-kinase by insulin and vanadate and the inhibitory effect of wortmannin. Rat adipocytes were preincubated for 20 min at 37 C with the indicated concentration of vanadate, insulin, or wortmannin, and PI3-kinase activity was then measured in anti-PTyr immunoprecipitates incubated with [γ-^{32}P]ATP and phosphatidylinositol (see *Materials and Methods*). The lipid product of PI3-kinase (PI3-P) was extracted and separated by TLC. B, Concentration-dependent activation of PI3-kinase by vanadate. Adipoctes were incubated for 20 min at 37 C in the absence and presence of the indicated concentrations of vanadate. PI3-kinase activity was then measured as described above.

inhibiting lipolysis (IC_{50} = 20 ± 2, 10 ± 0.9, and 8 ± 0.7 μM for vanadate, $VOSO_4$, and VO(acac)$_2$, respectively; Table 1), and vanadyl-dipicolinate was about 5 times less potent than vanadyl. This was followed by zinc (IC_{50} = 110 μM). Tungstate and molybdate were weakly antilipolytic.

Inhibition of adipose PTPases: possible relationships to antilipolysis

The adipose cell contains several members of the PTPase family (Elberg, G., *et al.*, manuscript in preparation). To obtain some clues about the putative PTPases involved in antilipolysis, fresh rat adipocytes were broken and fractionated into cytosolic (40,000 × *g* supernatant) and intrinsic plasma

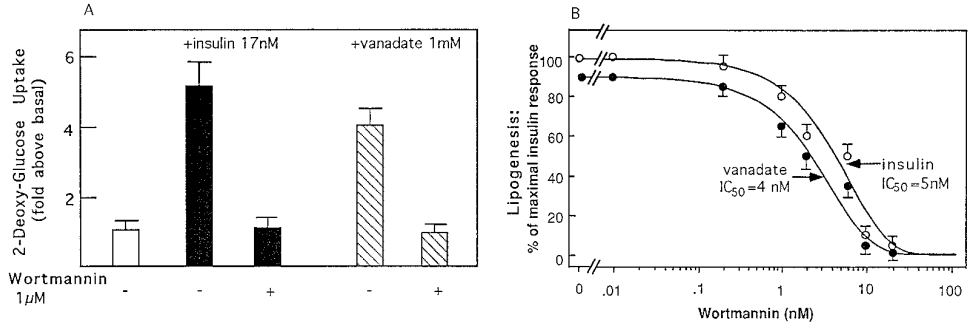

FIG. 3. Effect of wortmannin on inhibiting hexose uptake and lipogenesis activated by either insulin or vanadate. A, Adipocytes (2×10^6 cells/ml suspended in KRBH buffer-3% BSA) were incubated for 5 min at 37 C in the presence and absence of 1 μM wortmannin and then for 30 min at 37 C with or without vanadate or insulin. Aliquots (70 μl) were transferred to prewarmed tubes containing 2-D-[1,2-^{3}H]deoxyglucose (50,000 cpm/nmol; final concentration, 0.1 mM). Basal and activated uptake velocities were measured for 3 min. Termination was achieved by 0.1 μM phloretin and was followed by separating the adipocytes for counting over a layer of silicone oil (30). B, Concentration-dependent inhibition of insulin- or vanadate-activated lipogenesis by wortmannin. Adipocytes ($\sim 3 \times 10^5$ cells/ml) were divided into plastic vials (0.5 ml/vial) and incubated for 60 min at 37 C with 0.2 mM [U-^{14}C]glucose in either the presence or absence of insulin (17 nM), vanadate (1 mM), and the indicated concentrations of wortmannin. The amount of radioactivity incorporated into fat was then determined (29). Results are expressed as a percentage of the maximal stimulation obtained at 17 nM insulin. In all experiments, insulin-stimulated lipogenesis was 4- to 5-fold higher than basal (basal, $\sim$2,000 cpm/3 $\times$ 10^5 cells·h; insulin-stimulated, 8,000–10,000 cpm/3 $\times$ 10^5 cells·h).

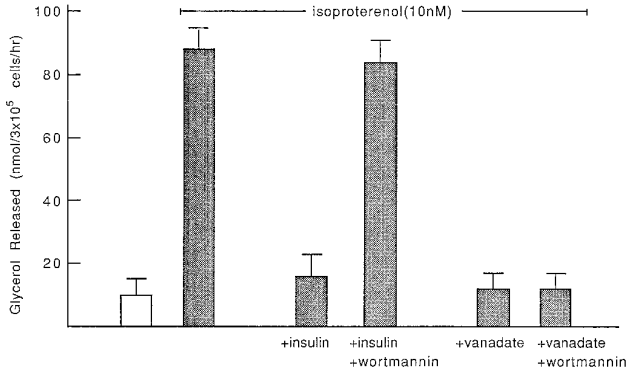

FIG. 4. Lack of inhibitory effect of wortmannin on the antilipolytic action of vanadate. Lipolysis was allowed to proceed for 1 h at 37 C in the absence or presence of isoproterenol (10 nM), insulin (17 nM), or vanadate (0.3 mM). Where indicated, the cells were incubated with 1 μM wortmannin for 10 min at 37 C before the addition of isoproterenol, vanadate, or insulin. The results are the mean ± SEM of four experiments.

membrane (Triton-soluble) PTPase fractions. The PTPase-inhibitory activities of the various vanadium compounds, Zn^{2+}, molybdate, and tungstate were examined for each fraction individually. Assuming comparable efficiencies of permeation into cell interiors, antilipolysis correlates better to the cell-free inhibition of membranal PTPases. The correlation plot between these two parameters gave a straight line (Fig. 5) and was best exemplified by $ZnSO_4$. Zinc sulfate exhibited intermediate potency in inhibiting lipolysis (IC_{50} value = 110 μM; Table 1) and was about 12 times less potent an inhibitor of membranal than of cytosolic PTPases (Table 1).

To evaluate further whether functional differences do exist between these two PTPase categories, we treated these fractions with the cysteinyl-specific reagent 2,2′-dithiodipyridine. After preincubation (final concentration, 2 mM; 20 min; 25 C), cytosolic PTPase activity was decreased by 5.5-fold. In contrast, membranal PTPase activity was not significantly reduced (our manuscript in preparation).

TABLE 1. Inhibition of isoproterenol-mediated lipolysis by various compounds in intact adipocytes and their potencies to inhibit membranal and cytosolic PTPases in cell-free experiments

Compound	Inhibition of lipolysis	Inhibition of adipose-protein PTyr-phosphatases (cell-free system)[a]	
	Intact cells, IC_{50} (μM)	Membranal IC_{90} (μM)[b]	Cytosolic
$VO(acac)_2$ (+4)	8 ± 0.7	0.22 ± 0.02	0.17 ± 0.015
$VOSO_4$ (+4)	10 ± 0.9	0.2 ± 0.02	0.18 ± 0.015
$NaVO_3$ (+5)	20 ± 2.0	0.22 ± 0.02	0.19 ± 0.02
VOdipic (+4)	50 ± 5	0.21 ± 0.02	0.20 ± 0.02
Zn sulfate (Zn^{+2})	110 ± 8	2.1 ± 0.15	0.17 ± 0.015
Tungstate (WO_4^{+3}) (+5)	2000 ± 150	20 ± 3	17 ± 2
Molybdate (MoO_4) (+6)	5000 ± 400	50 ± 4	31 ± 4

[a] The substrate for PTPase is [^{32}P]Poly-Glu_4, Tyr (experimental section).

[b] IC_{90} rather than IC_{50} values were used to minimize experimental errors originating from noncomplete inhibition at high concentrations of inhibitor.

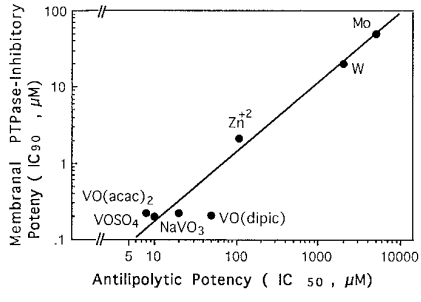

FIG. 5. A correlation plot among the potencies of the various compounds studied here to inhibit lipolysis in intact adipocytes and their efficacies to inhibit membranal PTPase in cell-free experiments. The values of IC_{50} (inhibition of lipolysis) and IC_{90} (inhibition of PTPases) are also summarized in Table 1.

Discussion

Vanadate mimics many of the bioeffects of insulin *in vivo* and *in vitro*, including antilipolysis, an inhibitory effect that is mechanistically distinct from the stimulating effects of the hormone on glucose and fat metabolism (42–44). After a

systematic study, we suggest that vanadate acts through an insulin-independent biochemical pathway. This finding *per se* is of pathophysiological interest, as it shows that insulin-responsive cells possess the machinery for promoting antilipolysis through an alternative route. Inhibition of lipolysis in adipose tissue relates physiologically and biochemically to the inhibition of glycogenolysis in muscle and liver (45); both effects are manifested by vanadate as well as insulin (46–48). We also suggest that vanadate-induced antilipolysis occurs at a site outside the lipolytic pathway, as lipolysis is inhibited regardless of the mode by which it is triggered. Unlike vanadate, insulin cannot overcome aberrations in the lipolytic cascade that result in high cAMP levels, inhibited isobutylmethylxanthine-sensitive cAMP phosphodiesterase activity, or arrested protein phosphatase 2A activity (14–16).

Attention should be given here to PI3-kinase, which, when activated by insulin, appears to be crucial for virtually all of its metabolic effects, including inhibition of lipolysis (33). In this study we show for the first time that vanadate also activates PI3-kinase. However, whereas wortmannin blocked PI3-kinase activation manifested by insulin and vanadate, it reversed only the antilipolytic effect of insulin and not that of vanadate. The activating effects of vanadate on hexose uptake and glucose metabolism were arrested by wortmannin, which adds weight to the suggestion that the lipid products of PI3-kinase activity participate in the recruitment of glucose transporter-4-containing vesicles to the plasma membranes (49). Overall, the results imply that the antilipolytic effect of vanadate is unrelated to PI3-kinase activity, providing yet more evidence that this physiologically important bioeffect can operate via an entirely insulin-independent mechanistic pathway.

How, then, does vanadate inhibit lipolysis? Data accumulated in our laboratory concerning the metalooxide effects on glucose and fat metabolism suggest that all of the insulin-like effects of vanadate may be initiated by inhibiting cellular protein PTPases (21–23, 25, 26). Inhibition of PTPases, in theory, allows unchecked autophosphorylation and activation of PTKs (50). This can initiate cascades by increasing the phosphorylation of substrates that otherwise are dephosphorylated by noninhibited cellular PTPases. The staurosporine-sensitive CytPTK, which is activated by vanadate, does not appear to participate in antilipolysis (22). However, another vanadate-activated nonreceptor PTK, which is less sensitive to inhibition by staurosporine, also exists in rat adipocytes (our manuscript in preparation).

In this study, we obtained experimental evidence suggesting that inhibition of membranal, rather than cytosolic, PTPases are required for the antilipolytic effect of vanadate. In cell-free experiments, vanadate is nearly equipotent in inhibiting both membranal and cytosolic PTPases, but this does not apply to the intact cell system. This is concluded from the sensitivity of CytPTK (21, 22) and other nonreceptor PTK (our manuscript in preparation) to activation by vanadate, whereas the InsRTK remains insensitive (20–22, 51, 52). Thus, the putative InsRTK-PTPase is resistant to inhibition by vanadate. In fact, a key, as yet unsolved, problem is whether there is a basic difference between cytosolic and membranal PTPases other than the latter being embedded (and, therefore, localized) into the plasma membrane. The issue begins to be resolved in this study. Both PTPase categories share considerable sequence homology, including the signature motif HCxxGxxR, with the low pKa-cysteine residue required for catalysis (53–56). Also, all PTPases have three acidic groups, two of which must be protonated and one nonprotonated for substrate binding and/or catalysis (53, 56). Although cytosolic and membranal PTPase fractions cannot be considered homogeneous preparations, we found that zinc is a ~12-fold more potent inhibitor of cytosolic PTPases and 2,2-dithiodipyridine inactivates cytosolic, but not membranal, PTPases. This reagent activates CytPTK in the 40,000 × *g* supernatant fraction, but not the membranal PTKs (not shown). Thus, there is clearly a difference between membranal and cytosolic PTPases, and it is at the level of substrate binding or catalysis. It is interesting to note that basic differences between cytosolic and membranal PTKs have been recently documented. Sonyang and associates found that CytPTKs prefer peptide substrates with Ileu or Val at the −1 position to the phosphorylated tyrosine, and Glu, Gly, or Ala at the +1 position. In contrast, the receptor family PTKs select peptides with Glu at the −1 position and large hydrophobic amino acids at the +1 position (57). We have recently found that staurosporine is a specific marker for CytPTK, being ~100-fold more potent an inhibitor of CytPTKs than intrinsic receptor and nonreceptor membranal PTKs (58). It is, therefore, conceivable that such a basic difference exists within the PTPase family, which opposes the intracellular effects of the PTKs.

Acknowledgments

We thank Rona Levin, Malka Kopelowitz, and Elana Friedman for typing this manuscript; Dr. Sandra Moshonov for critical reading; and Dr. Debbie C. Crans (Department of Chemistry, Colorado State University) for helpful suggestions and discussions.

References

1. **Meisner H, Carter Jr J** 1977 Regulation of lipolysis in adipose tissue. Horiz Biochem Biophys 4:91–103
2. **Steinberg D, Mayer SE, Khoo JC, Miller EA, Miller RE, Fredholm B, Eichner R** 1975 Hormonal regulation of lipase, phosphorylase and glycogen synthase in adipose tissue. Adv Cyclic Nucleotide Res 5:549–556
3. **Huttunen JK, Steinberg D, Mayer SE** 1970 Protein kinase activation and phosphorylation. Biochem Biophys Res Commun 41:1350–1357
4. **Egan J, Greenberg AS, Chang MK, Wek SA, Moos MC, Londos C** 1992 Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. Proc Natl Acad Sci USA 89:8537–8541
5. **Stock K, Prilop M(** 1974 Dissociation of catecholamine-induced formation of 3′-5′-monophosphate and release of glycerol in fat cells by prostaglandins E_1, E_2 and N^6-phenylisopropyladenosine. Arch Pharmacol Exp Pathol 282:15–31
6. **Trost T, Stock K** 1977 The affects of adenosine derivatives on cAMP accumulation and lipolysis in rat adipocytes and on adenylate cyclase in adipocyte plasma membranes. Arch Pharmacol 299:33–41
7. **Fain JN, Malbon CC** 1979 Regulation of adenylate cyclase by adenosine. Mol Cell Biochem 25:143–149
8. **Manganiello VC, Vaughan M** 1973 Ion effect of insulin on cyclic adenosine 3′:5′ monophosphate phosphosdieterase activity in fat cells. J Biol Chem 248:7164–7170
9. **Zinman B, Hollenberg CH** 1974 Effect of insulin on cyclic adenosine 3′:5′ monophosphate phosphodiesterase activity in fat cells. J Biol Chem 249:2182–2187
10. **Kono T, Barham T** 1973 Effects of insulin on the levels of adenosine 3′:5′-monophosphate and lipolysis in isolated rat fat cells. J Biol Chem 248:7417–7426
11. **Kono T, Robinson FW, Sarver JA** 1975 Insulin-sensitive phosphodiesterase. Its localization, hormonal stimulation, and oxidative stabilization. J Biol Chem 250:7826–7835
12. **Olsson H, Belfrage P** 1987 The regulatory and basal phosphorylation sites of

hormone-sensitive lipase are dephosphorylated by PP-1, PP2A and PP-2C, but not by PP-2B. Eur J Biochem 168:399–405
13. **Wood LS, Emmison N, Borthwick C, Yeaman SJ** 1993 The protein phosphatases responsible for dephosphorylation of hormone-sensitive lipase in isolated rat adipocytes. Biochem J 295:531–535
14. **Shechter Y** 1982 Evaluation of adenosine or related nucleosides as physiological regulators of lipolysis in adipose tissue. Endocrinology 110:1579–1583
15. **Shechter Y** 1984 Differential effects of two phosphodiesterase inhibitors on fat cell metabolism. Endocrinology 115:1787–1791
16. **Shisheva A, Shechter Y** 1991 Effect of okadaic acid in rat adipocytes. Differential stimulation of glucose and lipid metabolism and induction of refractoriness to insulin and vanadate. Endocrinology 129:2279–2288
17. **Cohen P, Holmes CFB, Tsukitani Y** 1990 Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci 15:98–102
18. **Degani H, Gochin M, Karlish SJD, Shechter Y** 1981 Electron paramagnetic studies and insulin-like effects of vanadate in rat adipocytes. Biochemistry 20:5795–5799
19. **Shechter Y** 1990 Insulin-mimetic effects of vanadate. Diabetes 39:1–5
20. **Shisheva A, Shechter Y** 1992 Quercetin selectively inhibits insulin receptor function *in vitro* and the bioresponses of insulin and insulinomeimetic agents in rat adipocytes. Biochemistry 31:8059–8063
21. **Shisheva A, Shechter Y** 1992 A cytosolic protein tyrosine kinase in rat adipocytes. FEBS Lett 300:93–96
22. **Shisheva A, Shechter Y** 1993 Role of cytosolic tyrosine kinase in mediating insulin-like actions of vanadate in rat adipocytes. J Biol Chem 268:6463–6469
23. **Elberg G, Li J, Shechter Y** 1994 Vanadium activates or inhibits receptor and non-receptor protein tyrosine kinases in cell-free experiments, depending on its oxidation state. J Biol Chem 269:9521–9527
24. **Lau KHW, Farley JR, Baylink DJ** 1989 Phosphoryrosyl protein phosphatases. Biochem J 257:23–26
25. **Li J, Elberg G, Libman J, Shanzer A, Gefel D, Shechter Y** 1995 Insulin-like effects of vanadate, tungstate and molybdate; mediation through insulin receptor independent pathways. Endocr J 3:631–637
26. **Li J, Elberg G, Gefel D, Shechter Y** 1995 Permolybdate and pertungstate-potent stimulators of insulin effects in rat adipocytes: mechanism of action. Biochemistry 34:6218–6225
27. **Bersted BH, Belford RL, Paul IC** 1968 Crystal and molecular structure of orthorthrombic vanadyl (IV) 2,6-dicarboxylate tetrahydrate. Inorg Chem 17:1557–1566
28. **Rodbell M** 1964 Metabolism of isolated fat cells. I. Effects of hormone on glucose metabolism and lipolysis. J Biol Chem 239:375–380
29. **Moody AJ, Stan MA, Stan M, Glieman J** 1974 A simple free fat cell bioassay for insulin. Horm Metab Res 6:12–16
30. **Whitesell RR, Glieman J** 1979 Kinetic parameters of transport of 3-*O*-methylglucose in adipocytes. J Biol Chem 254:5276–5283
31. **Shisheva A, Shechter Y** 1993 Mechanism of pervanadate stimulation and potentiation of insulin-activated glucose transport in rat adipocytes: dissociation from vanadate effect. Endocrinology 133:1562–1568
32. **Swarup G, Subratumanyam G** 1989 Purification and characterization of a protein-phosphotyrosine phosphatase from rat spleen which dephosphorylates and inactivates a tyrosine-specific kinase. J Biol Chem 264:7801–7808
33. **Okada J, Kawano Y, Sakakibara T, Hazeki O, Ui M** 1994 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
34. **Kelly KL, Ruderman NB** 1992 Phosphatidylinositol-3-kinase in isolated rat adipocytes. J Biol Chem 268:3423–3428
35. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254
36. **Gilman AG** 1987 G. proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615–649
37. **Tang W-J, Gilman AG** 1992 Adenylyl cyclases. Cell 70:869–872
38. **Haystead TAJ, Sim ATR, Carling D, Honnor RC, Tsukitani Y, Cohen P, Hardie DG** 1989 Effects of tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337:78–81
39. **Kelly KL, Ruderman NB** 1993 Insulin-stimulated phosphatidylinositol 3-kinase. J Biol Chem 268:4391–4398
40. **Heller-Harrison RA, Morin MM, Guilherme A, Czech MP** 1996 Insulin-mediated targeting of phosphatydylinositol 3-kinase to GLUT4-containing vesicles. J Biol Chem 271:10200–10204
41. **Shechter Y, Ron A** 1986 Effect of depletion of phosphate and bicarbonate ions on insulin action in rat adipocytes. J Biol Chem 261:14951–14954
42. **Czech MD** 1977 Molecular basis of insulin action. Annu Rev Biochem 46:357–381
43. **Shechter Y** 1985 Studies on insulin receptors: implications for insulin action. In: Conn MP (ed) The Receptors. Academic Press, Orlando, vol 2:221–244
44. **Shechter Y, Yaish P, Chorev M, Gilon H, Braun S, Levitzki A** 1989 Inhibition of insulin-dependent lipogenesis and anti-lipolysis by protein-tyrosine kinase inhibitors. EMBO J 8:1671–1676
45. **Belfrage P, Donner J, Stralfors P (eds)** 1987 Mechanism of Insulin Action. Elsevier (Biomedical Division), Amsterdam
46. **Gil J, Miralpeix M, Carreras J, Bartrons R** 1988 Insulin-like effects of vanadate on glucokinase activity and fructose 2.6-bisphosphate levels in the liver of diabetic rats. J Biol Chem 263:1868–1871
47. **Mortimore GE** 1963 Effect of insulin on release of glucose and urea by isolated rat liver. Am J Physiol 204:699–704
48. **Bruck R, Prigozin H, Krepel Z, Rotenberg P, Shechter Y, Bar-Meir S** 1991 Vanadate inhibits glucose output from isolated perfused rat liver. Hepatology 14:540–544
49. **Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR** 1994 Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp 70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol Cell Biol 14:4902–4911
50. **Srivastava A** 1990 Non-receptor protein tyrosine kinases of normal tissues. Int J Biochem 11:1229–1234
51. **Fantus GI, Kadota S, Deragon G, Foster B, Posner BI** 1989 Pervanadate [peroxide(s) of vanadate] mimics insulin action in rat adipocytes via activation of the insulin receptor tyrosine kinase. Biochemistry 28:8864–8871
52. **Strout HV, Vicario PP, Superstein R, Slater EE** 1989 The insulin-mimetic effect of vanadase is not correlated with insulin receptor tyrosine kinase activity, nor phosphorylation in mouse diaphragm *in vivo*. Endocrinology 124:1918–1924
53. **Guan KL, Dixon JE** 1991 Evidence for protein tyrosine phosphatase catalysis proceeding via a cyteine-phosphate intermediate. J Biol Chem 266:17026–17030
54. **Cho H, Krishnaraj R, Kitas E, Bannwarth W, Walsh CT, Anderson KS** 1992 Isolation and structure elucidation of a novel phosphocystein intermediate in the LAR protein tyrosine phosphatase enzymatic pathway. J Am Chem Soc 114:7296–7298
55. **Zhang Z-Y, Wang Y, Dixon JE** 1994 Dissecting the catalytic mechanism of protein tyrosine phosphatase. Proc Natl Acad Sci USA 91:1624–1627
56. **Bradford D, Flint AJ, Tonks NK** 1994 Crystal structure of human protein tyrosine phosphatase. Science 263:1397–1404
57. **Songyang Z, Carraway KL, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Bubbard SR, Smith DP, Eng C, Lorenzo MR, Ponda BA, Mayer BJ, Cantley LC** 1995 Catalytic specificity of protein tyrosine kinase is critical for selective signalling. Nature 373:536–539
58. **Elberg G, Li J, Leibovitch A, Shechter Y** 1995 Non-receptor cytosolic protein tyrosine kinase from various rat tissues. Biochim Biophys Acta 1269:299–306