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Insulin-like effects of tungstate and molybdate: mediation through insulin receptor independent pathways

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The insulin-like effects of tungstate (W) and molybdate (Mo) were studied in rat adipocytes and compared to those of vanadate. Other than being less potent, W and Mo resembled vanadate in stimulating lipogenesis, in activating glucose oxidation, in enhancing rate of hexose uptake, and in inhibiting lipolysis. Tungstate and molybdate did not activate the insulin-receptor tyrosine kinase (InsRTK). Quercetin which blocks InsRTK activity and insulin stimulation of glucose metabolism, failed to inhibit when these bioeffects were stimulated by W or Mo. The metalooxide, however, activated a staurosporine sensitive non receptor, cytosolic protein tyrosine kinase (CytPTK), and staurosporine blocked W or Mo dependent lipogenesis in rat adipocytes. Staurosporine did not prevent Mo and W either from activating hexose transport, or from inhibiting lipolysis. Tungstate and molybdate were less effective than vanadate in inhibiting adipose PTPases in cell free systems. Membranal PTPases were more sensitive to W and Mo inhibition than cytosolic PTPases. While the presence of a nucleophile such as hydroxylamine reversed inhibition of PTPase by vanadate it did not affect inhibition by W or Mo. In summary, the insulinomimetic effects of W and Mo appear to resemble qualitatively that of vanadate in all respects. Both act in an insulin receptor-independent-fashion, activate CytPTK and trigger additional effects that are not mediated by the InsRTK or by CytPTK. The quantitative differences may be attributed to reduced capacity of W and Mo relative to vanadate to inhibit the relevant PTPases in intact cells.

Keywords: insulin effects; insulin mimetic; alternative pathways; metalooxides; rat adipocytes

Introduction

Vanadate and vanadyl mimic virtually all the biological effects of insulin in a large variety of insulin responsive tissues (reviewed by Shechter, 1990). In rat adipocytes vanadate enhances rate of hexose uptake (Dubyak & Kleinzeller, 1980), stimulates glucose and fat metabolism (Shechter & Karlish, 1980; Shechter & Ron, 1986), activates glycogen synthase (Tamura *et al.*, 1984) and inhibits isoproterenol mediated lipolysis (Degani *et al.*, 1981).

The mechanism by which vanadate mimics the actions of insulin is still unknown. In vanadate pretreated cells and tissues the insulin receptor is not, or is only negligibly activated (Fantus *et al.*, 1989; Mooney *et al.*, 1989; Strout *et al.*, 1989; Venkatesan *et al.*, 1991). This and other studies suggest that the effects of vanadate are not mediated through the insulin receptor (Green, 1986; Shisheva & Shechter, 1992a,b). Recently, a 53 kDa non-receptor cytosolic protein tyrosine kinase (CytPTK) was identified in rat adipocytes (Shisheva & Shechter, 1993). Unlike with the InsRTK, CytPTK is activated 3-5-fold in vanadate pretreated cells. The enzyme is inhibited by very low concentrations of staurosporine (ID_{50} = 3 nM)*, which also prevented vanadate from stimulating glucose metabolism. Staurosporine, however, neither inhibited vanadate from activating hexose uptake nor antagonized its antilipolytic effect (Shisheva and Shechter, 1993). The outcome of these findings is that vanadate can facilitate additional insulin like effects which are not mediated via CytPTK, or InsRTK but by mechanisms that are presently obscure.

Vanadate and vanadyl inhibit protein phosphotyrosine phosphatases (PTPases, reviewed in Lau *et al.*, 1989). Since many of the non-receptor PTKs are activated by self-phosphorylation on tyrosine moieties (reviewed by Srivastava, 1990), we have postulated that the activation of CytPTK may be secondary to inhibition of PTPases. The ability of vanadate to activate CytPTK was preserved also after cell disintegration (Elberg *et al.*, 1994). Vanadate activated CytPTK and inhibited PTPase activity in the adipocytic 40 000 *g* supernatant fraction with half-maximal effects at 3 ± 0.3 μM (Elberg *et al.*, 1994 and in preparation). Since the CytPTK, but not the InsRTK is activated in vanadate pretreated adipocytes, we further hypothesized the presence of both vanadate sensitive and vanadate insensitive PTPases within the same cell (in preparation).

Molybdate (Mo) and tungstate (W) also inhibit PTPases in some tissues (reviewed in Lau *et al.*, 1989). Less is known about their inhibitory mechanisms, inhibitory potencies and whether or not they inhibit the same PTPase species as vanadate and vanadyl. In the cell free adipocytic experimental system, W and Mo resembled vanadate in activating CytPTK, although higher concentrations were required (Elberg *et al.*, 1994). Our purpose now was to study systematically whether W and Mo are insulinomimetic in the adipose cell and if so whether they do so via the insulin-dependent or the insulin-independent (vanadate-dependent) biochemical pathways.

Results

Insulin-like effects of the metalooxides in rat adipocytes

Lipogenesis The ability of tungstate and molybdate to activate CytPTK in cell-free experiments suggested that these metalooxides could produce the biological effects of insulin in intact cells as well. This is provided that they can traverse the plasma membrane and permeate into the cell interior. Indeed, both tungstate and molybdate, following preincubation with the cells, markedly stimulated lipogenesis (Figure 1). ED_{50} values were 0.1 ± 0.02, 7.0 ± 0.3 and 11 ± 1.4 mM for vanadate, tungstate and molybdate†, respectively. Fluoride

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*Staurosporine has no inhibitory or stimulatory effect on rat-adipocytic PTPases (unpublished observations).

†Maximal stimulation with Mo was 53 ± 4% (see below). This was considered as maximal response in calculating the ED_{50} value.

had no effect on lipogenesis. Maximal stimulations compared to insulin response were 120 ± 7% for vanadate, 97 ± 5% for tungstate and 53 ± 4% for molybdate and were significantly dependant upon whether or not the cells were preincubated with the metalooxides prior to the assay (Table 1).

Stimulation of lipogenesis was further investigated by analysing the incorporation of either [1-^{14}C]glucose or [6-^{14}C]glucose into lipids. The former is an index of the glycerol-phosphate incorporation into lipids, whereas the latter represents synthesis of fatty acids. Tungstate and molybdate activated both biochemical pathways. No significant differences could be observed in this respect between the activating effects of W or Mo to that of insulin or vanadate (Figure 2).

Effects on glucose oxidation Tungstate and molybdate stimulated oxidation of glucose to CO_2 (Figure 3). The stimulating effects were both on [6-^{14}C]glucose oxidation to $^{14}CO_2$, an index of glycolysis, and on $^{14}CO_2$ production from [1-^{14}C]glucose, an index of the pentose phosphate pathway. The metalooxides resembled insulin (and vanadate) in their ability to stimulate glucose oxidation both via glycolysis and the hexosemonophosphate pathways.

Stimulation of hexose influx It was our further intention to determine the effects of the metalooxides on glucose entry, utilizing the non-metabolizable-glucose analog 3-0-methyl glucose. Following preincubation, tungstate and molybdate stimulated glucose entry to nearly the same extent as did insulin or vanadate (Figure 4). Both had been previously observed to stimulate 3-0-methyl-glucose uptake into rat adipocytes (Goto *et al.*, 1992). Compared to their effects on lipogenesis (Figure 1) tungstate and more so molybdate had a greater effect on glucose entry. Previously, we observed that the activating effect of vanadate on hexose transport was not quenched by staurosporine (Shisheva & Shechter, 1993). We concluded that vanadate stimulation of hexose influx is not mediated by CytPTK. The same also holds true for tungstate and molybdate. Staurosporine did not inhibit the activating effects produced by those metalooxides (Table 2).

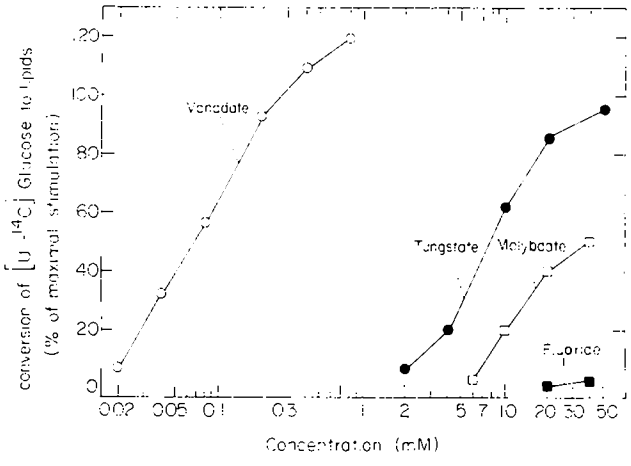

Figure 1 Concentration dependent stimulation of lipogenesis by the metalooxides. Freshly prepared adipocytes (2×10^4 cells/ml suspended in KRB buffer containing 0.7% BSA) were preincubated for 20 min with the indicated concentrations of the metalooxides. The cells were then supplemented with [U-^{14}C]glucose (final concentration 0.16 mM) and lipogenesis was performed for an additional 1 h at 37°C. Results shown are taken from a typical representative experiment in which insulin response was 5–7 fold the basal level. Maximal response (100%) is that obtained in the presence of 17 nM insulin

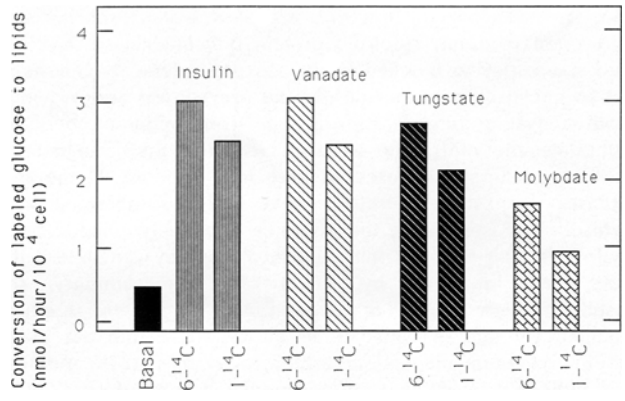

Figure 2 Stimulation by insulin and metalooxides of [1-^{14}C]glucose and [6-^{14}C]glucose incorporation into lipids. Lipogenesis was performed for 1 h at 37°C in the absence or the presence of insulin (17 nM), vanadate (1 mM), tungstate (40 mM) or molybdate (40 mM). The cells were preincubated with the metalooxides for 20 min at 37°C prior to adding 0.16 mM of either [1-^{14}C]- or [6-^{14}C]glucose (2500 c.p.m./ml). Incorporation of [1-^{14}C]glucose and [6-^{14}C]glucose into lipids represent glycerol phosphate, and fatty acid pathways respectively

Table 1 Effect of preincubation on tungstate and molybdate induced lipogenesis

Additions conversion to lipids[a] *nmol/h/10⁴ cells*	*[U-^{14}C]glucose stimulation*	*% Maximal insulin*
Basal	2.0±0.1	0
Insulin (17 nM)	8.3±0.2	100
Vanadate (1 mM) (no preincubation)	6.4±0.15	70
Vanadate (1 mM) (preincubation 20 min, 37°C)[b]	9.6±0.2	120
Tungstate (40 mM) (no preincubation)	4.2±0.1	35
Tungstate (40 mM) (preincubation – 20 min, 37°C)[b]	8.1±0.1	97
Molybdate (40 mM) (no preincubation)	3.0±0.1	16
Molybdate (40 mM) (preincubation – 20 min, 37°C)	5.35±0.15	53

[a]Assay of lipogenesis was performed for 1 h at 37°C. [b]Cells were preincubated for 20 min at 37°C in the absence and the presence of the indicated concentrations of metalooxides or insulin. The cells were then supplemented with [U-^{14}C]glucose, and subjected to lipogenesis for an additional hour at 37°C

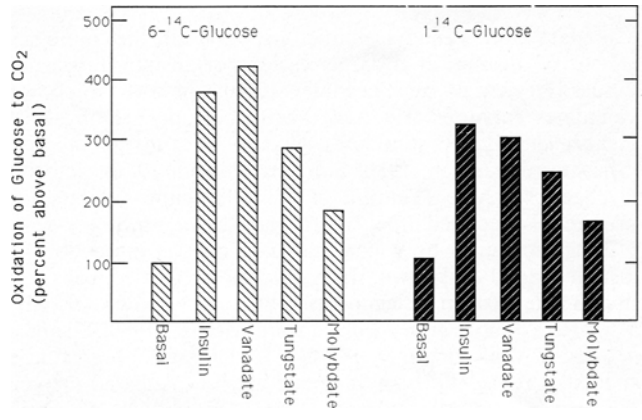

Figure 3 Metalooxide-dependent stimulation of glucose oxidation via (i) glycolysis, and (ii) pentose phosphate pathways. Cells were preincubated with none, vanadate (1 mM), tungstate (40 mM) and molybdate (40 mM) for 20 min at 37°C. The cells were then supplemented with either [6-^{14}C]glucose or with [1-^{14}C]glucose (final concentrations - 0.16 mM) and incubated for 1 h at 37°C. Maximal stimulation is that obtained with 17 nM insulin

Inhibition of lipolysis One of the key actions of insulin, not related to glucose metabolism, is the hormone's ability to antagonize lipolysis mediated by counter-regulatory hormones to insulin (Avruch *et al.*, 1972). Vanadate was shown previously to mimic this effect (Degani *et al.*, 1981). We have therefore examined here whether tungstate and molybdate have antilipolytic activity as well. Figure 5 shows that tungstate and molybdate strongly inhibit isoproterenol mediated lipolysis (97 ± 3% inhibition of lipolysis). Fifty percent inhibition is evidence at 0.1 ± 0.02 mM, 5 ± 0.2 and 7.0 ± 0.3 mM for vanadate, molybdate and tungstate, respectively, namely in the same concentration ranges the metalooxides stimulated half-maximal lipogenesis (Figure 1). Staurosporine did not reverse the antilipolytic effects (not shown). In fact, we have suggested in a previous study (with respect to insulin as well) that endogenous intracellular tyrosine phosphorylation (other than receptor autophosphorylation) may not be required for the expression of antilipolysis (Shechter *et al.*, 1989; Shisheva & Shechter, 1992b). Gotschalk (1991) suggested that endogenous tyrosine phosphorylation is not required for the insulin effect in activating pyruvate dehydrogenase. Both, inhibition of lipolysis and activation of pyruvate dehydrogenase are dependent on PSer/PThr dephosphorylation (Kahn & Shechter, 1990).

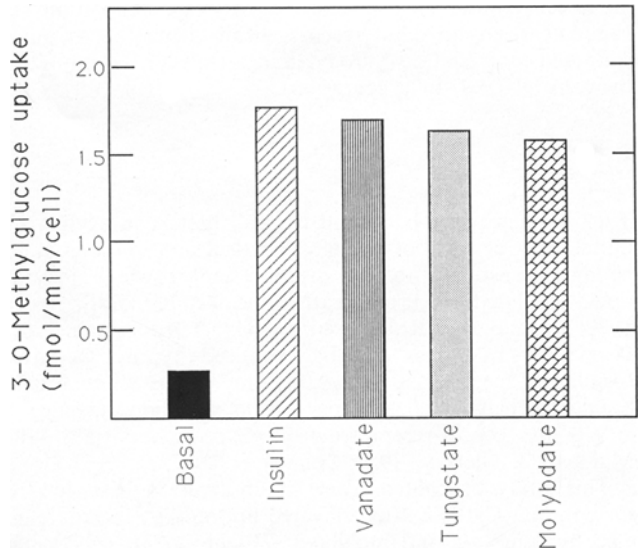

Figure 4 Stimulation of hexose transport by the metalooxides. Adipocytes (40% cell suspension in KRB buffer containing 1% BSA) were preincubated at 37°C for 30 min with none, insulin 17 nM; vanadate 1 mM; tungstate 20 mM or molybdate 40 mM. Aliquots (70 µl) were then transferred to tubes containing 3-0[^{14}C]methylglucose (50 000 c.p.m./nmol, final concentration 0.1 mM) and centrifuged through a layer of silicone oil

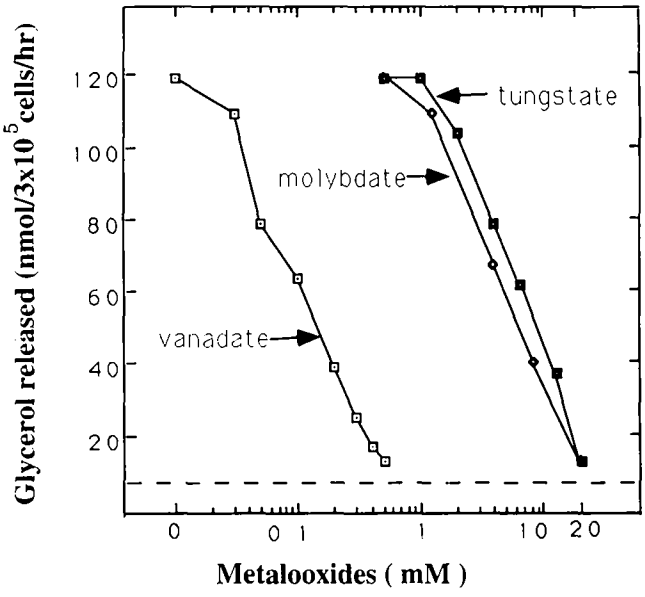

Figure 5 Dose-dependent inhibition of isoproterenol-mediated lipolysis by vanadate, molybdate and tungstate. Lipolysis was performed for 1 h at 37°C in the absence and the presence of the indicated concentrations of the three metalooxides and isoproterenol (final concentration 30 µM). Quantities of glycerol released were then determined. The horizontal dashed line indicates amount of glycerol released in the absence of isoproterenol

Table 2 Lack of staurosporine effect on metalooxides stimulated hexose uptake

Addition	*3-0-Methylglucose uptake*
fmol/min/cell	
Basal + Staurosporine	0.30 ± 0.02
	0.33 ± 0.03
Insulin (17 nM) + Staurosporine	1.70 ± 0.08
	1.60 ± 0.08
Tungstate (40 mM) + Staurosporine	1.65 ± 0.12
	1.70 ± 0.14
Molybdate (40 mM) + Staurosporine	1.55 ± 0.11
	1.50 ± 0.13

Adipocytes (40% cell suspension in KRB buffer, containing 1% BSA), were preincubated at 37°C for 30 min in the absence or presence of staurosporine (0.35 µM) and then for an additional 30 min with or without insulin or metalooxides or insulin. Aliquots (70 µl) were transferred into tubes containing 3-0-[methyl-^{14}C]glucose (50 000 c.p.m./nanomol, final concentration 0.1 mM). Basal uptake was measured for 15 s and either insulin- or metalooxide-activates uptake for 3 s. Phloretin (0.1 µM) was added for transport termination followed by centrifuging an aliquot through a silicone oil layer

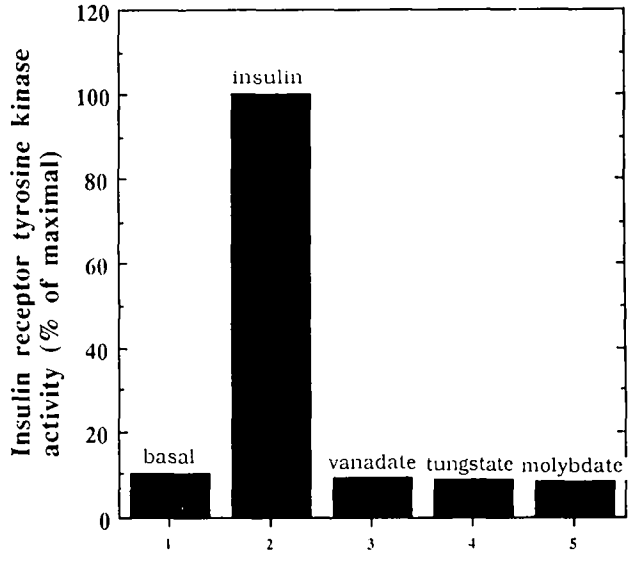

Figure 6 Lack of metalooxide effects on insulin receptor tyrosine kinase catalyzed PolyGlu$_4$ Tyr phosphorylation. The assay was performed for 20 min at 22°C in 60 µl of 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100, WGA purified rat liver insulin receptor (0.6 µg protein), 20 mM $MgSO_4$, 1 mM $MnCl_2$ 14 µM ATP with or without insulin (0.1 µM), vanadate (100 µM), tungstate (1 mM) or molybdate (1 mM). PolyGlu$_4$ Tyr was then added (final concentration of 90 µg/ml) and the reaction allowed to proceed for an additional 40 min at 22°C. Aliquots were withdrawn for determining phosphotyrosine content, as described under Materials and methods

Mechanism of action of tungstate and molybdate

Tungstate and molybdate do not mediate their effects via the InsRTK Like vanadate, tungstate and molybdate had neither stimulatory nor inhibitory effects on InsRTK-catalyzed PolyGlu$_4$Tyr phosphorylation in the cell-free system (Figure 6). With respect to intact cells: we have previously shown that quercetin inhibits InsRTK in cell-free assays, and prevents the stimulating effects of insulin, but not of vanadate on glucose metabolism in rat adipocytes (Shisheva & Shechter, 1992b). This proved to be valid also for tungstate and molybdate (Figure 7), which were slightly more effective in quercetin pretreated adipocytes. Thus the stimulating effects of these metalooxides are not mediated via the InsRTK.

Tungstate and molybdate mediate several of their effects on glucose metabolism via CytPTK As with vanadate, preincubation of rat adipocytes for 20 min at 37°C with tungstate and molybdate, stimulated CytPTK activity. Level of stimulation increased 3.3-, 2.5- and 2.3-fold for vanadate, tungstate and molybdate, respectively (Figure 8). also, staurosporine, a powerful inhibitor of CytPTK (Shisheva & Shechter, 1992a, 1993) fully blocked tungstate and molybdate dependent lipogenesis in rat adipocytes (Figure 7). Under the same experimental conditions, staurosporine had only a marginal effect on insulin stimulated lipogenesis (Figure 7).

Inhibition of adipocytic PTPases by molybdate and tungstate differs mechanistically from that of vanadate and vanadyl We have already reported that vanadate, tungstate and molybdate were able to stimulate CytPTK activity in adipocytic supernatant fractions in cell-free experiments. ED_{50} values were 3 ± 0.7, 20 ± 3 and $30 \pm 4\,\mu$M for vanadate tungstate and molybdate, respectively (Elberg *et al.*, 1994). The common denominator for all three metalooxides is their ability to inhibit PTPases (Lau *et al.*, 1989). To further ensure that CytPTK activation is connected to PTPase inhibition, the potency of all three metalooxides in inhibiting adipose PTPase activity was studied directly. Adipose PTPases were fractionated into cytosolic (40 000 *g* supernatant) PTPases, intrinsic plasma-membrane (Triton-soluble) PTPases and to glycoprotein (receptor-like) PTPases obtained by further purifying solubilized plasma-membranes on WGA-agarose affinity column. Each of these three fractions was examined individually. The results, summarized in Figure 9, show the following: (a) Vanadate is the most potent PTPase inhibitor and is nearly equipotent with respect to all three fractions examined. (b) Order of inhibitory potency is vanadate>> tungstate ≥ molybdate. (c) Unlike vanadate, tungstate and molybdate are poor inhibitors of cytosolic-PTPase(s) and (d) considerably more potent inhibitors of intrinsic plasma-membrane PTPases, both of the receptor and the non-receptor types. These findings correlate in general with the potency of the metalooxides to stimulate insulin bioeffects. Furthermore, the results suggest the existence of tungstate and molybdate sensitive and insensitive PTPases within the same tissue.

When PTPase-mediated hydrolysis of pNPP is performed in the presence of 100 mM hydroxylamine, PTPase activity is increased by about 2–3-fold (not shown). The presence of this nucleophile fully reversed the inhibitory action of vanadate (Figure 9, right panels). In contrast, the inhibitory actions of tungstate and molybdate, were not blocked at all by hydroxylamine and were even slightly more potent in its presence. Thus, the mode(s) of PTPase inhibition by vanadate seems to differ mechanistically from that of tungstate and molybdate. Hydroxylamine also reversed inhibition by vanadyl (+4, in preparation).

Discussion

There is a growing basic and clinical interest in agents that mimic the actions of insulin, particularly in compounds acting downstream to the insulin-receptor which may be down-regulated in certain pathological states (Kahn *et al.*, 1977; Kosmakos & Roth, 1980; Olefsky & Kolterman, 1981). If the insulin effects can be produced by an alternative (insulin-independent) pathway, the importance of such compounds is heightened considerably as 'insulin resistance' is largely due to post-receptor defects (Caro & Amatruda, 1980; Marshall & Olefsky, 1980; Garvey, 1989).

This study originated from an observation in a cell-free system, that CytPTK was activated not only by vanadate but also by tungstate and molybdate (Elberg *et al.*, 1994). The study followed two directions, the first aimed to determine whether activation of CytPTK in cell-free experiments is translated into facilitation of insulin effects in intact cells through the receptor-independent pathway and the second

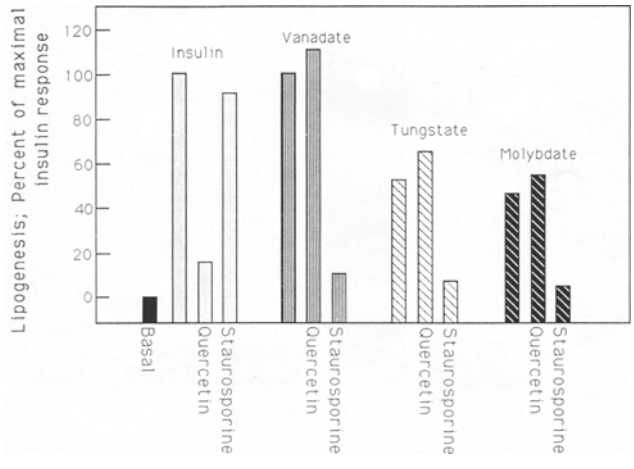

Figure 7 Effect of staurosporine and quercetin on metalooxides stimulated lipogenesis. Adipocytes (2×10^4 cells/ml) suspended in KRB buffer containing 0.7% BSA, were preincubated with either none, 0.3 μM staurosporine, or 100 μM quercetin for 20 min at 37°C and then with insulin (17 nM), vanadate (1 mM), tungstate (20 mM) or molybdate (40 mM) for an additional 20 min at 37°C. [U-^{14}C]glucose was then added (0.16 mM) and lipogenesis was allowed to proceed for 1 h at 37°C

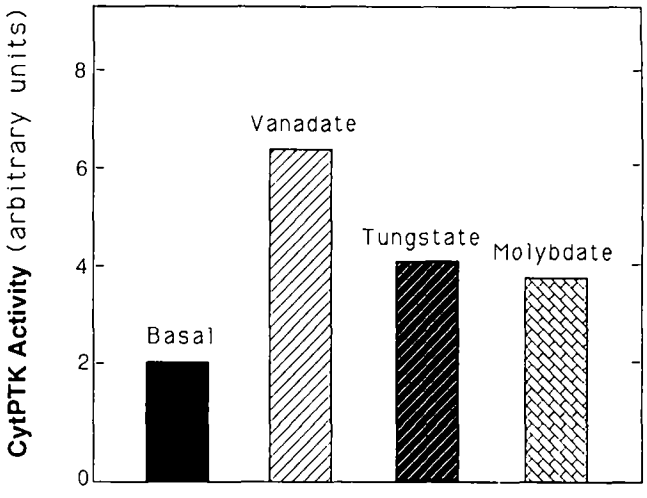

Figure 8 Stimulation of CytPTK activity by metalooxides in intact adipocytes: Freshly prepared rat adipocytes were preincubated in the presence or absence of vanadate (1 mM), tungstate (20 mM) or molybdate (40 mM) for 20 min at 37°C. The cells were then lyzed and the specific activities of CytPTK in the 40 000 × g supernatant fraction were determined

was aimed at evaluating the spatial relationships between PTPase inhibition and CytPTK activation. Tungstate and molybdate did indeed mimic the biological actions of insulin via an insulin-receptor-independent pathway (Figures 1–3). The metalooxides resembled vanadate also in facilitating the bioeffects, hexose influx and inhibition of lipolysis (Figures 4 and 5) that are independent of CytPTK (Shisheva & Shechter, 1992b, 1993). The basic difference compared to vanadate was quantitative rather than qualitative. This is in terms of the much higher concentrations of molybdate and tungstate that were required to facilitate their bioeffects and to inhibit PTPase activity. The fact that hydroxylamine was found to fully reverse vanadate inhibition of PTPase, while having no effect on that of tungstate and molybdate (Figure 9), may indicate that the mechanism of inhibition of PTPase by vanadate is not the same as that of tungstate or molybdate. It has been suggested that the hydrolysis of the phosphoenzyme intermediate (by H_2O) is the rate limiting step in catalysis of PTPases (Walton & Dixon, 1993). This probably explains the activation of PTPase by hydroxylamine (in preparation). It also further suggests that from enzymological stand point, vanadate inhibition occurs by further decreasing

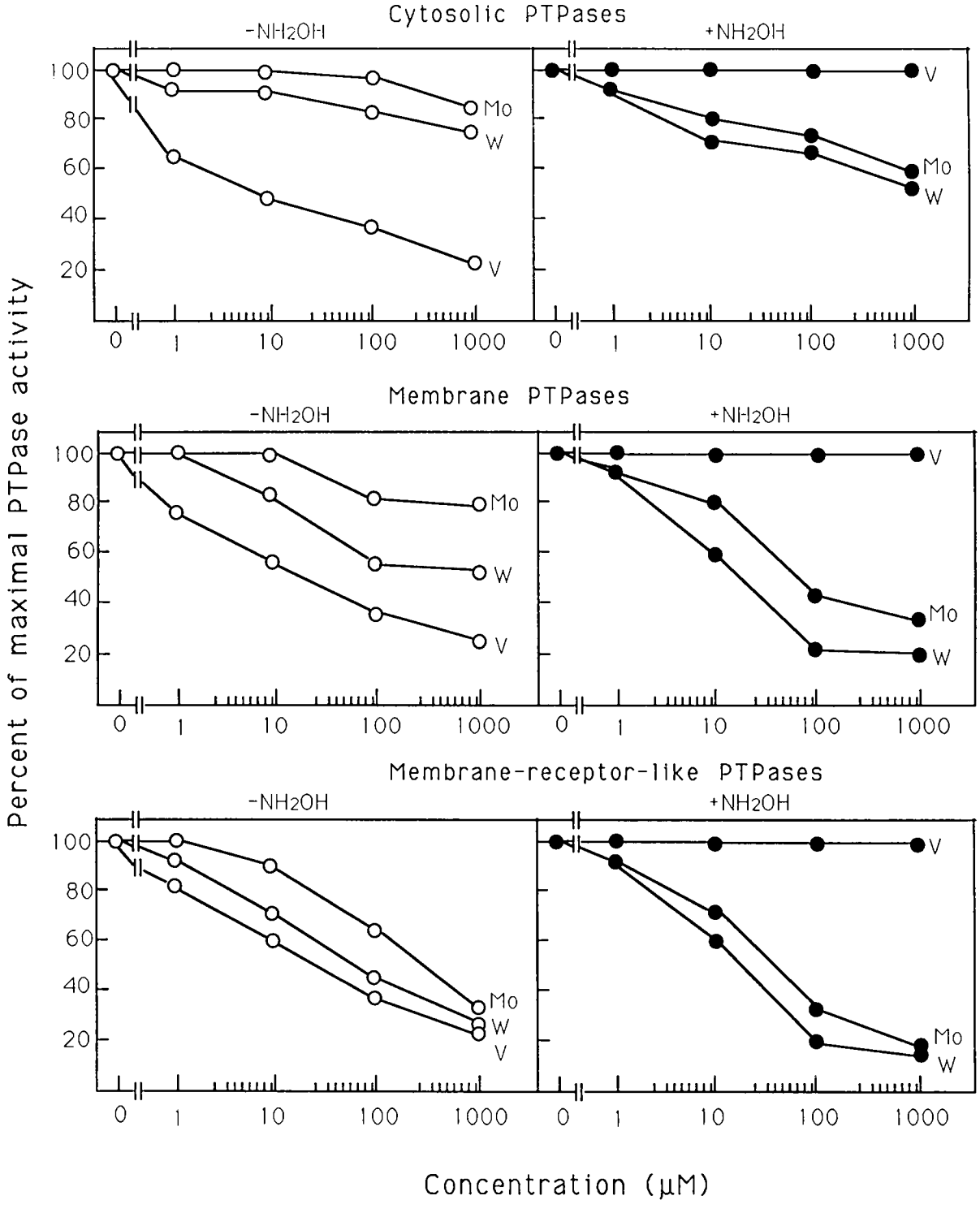

Figure 9 Concentration dependent inhibition of rat adipocytic PTPases by vanadate (V), molybdate (Mo) and tungstate (W). Three fractions of PTPases were studied; cytosolic PTPases (upper panels); triton-soluble plasma membrane-PTPase (middle panel), and receptor-like (glycoprotein) PTPases (bottom panels). The assay was performed in the absence and the presence of the indicated concentrations of the metalooxides, and in the absence or the presence of hydroxylamine (100 mM, right panels). 100% activity is that obtained in the absence of added inhibitors

hydrolysis, and hydroxylamine by accelerating this rate limiting step reverses the inhibition.

In summary, we show here that tungstate and molybdate are insulinomimetic agents in intact rat adipocytes and by virtually all criteria examined, they qualitatively resemble vanadate. The basic difference is that molybdate and tungstate inhibit PTPases through a different mechanism which appears to be less effective in the intact cell, therefore higher concentrations of these metalooxides are required. This issue is currently being studied in our laboratory.

Materials and methods

Materials

D-[U-^{14}C], D-[1-^{14}C], D-[6-^{14}C]glucose and D-3-0-[methyl-^{14}C]glucose were purchased from New England Nuclear (Boston, MA). Collagenase Type I (134 U/mg) was obtained from Worthington Biochemicals (Freehold, N.J.). Porcine insulin was purchased from Eli Lilly Co. (Indianapolis, IN). Phloretin, 3-0-methyl-D-glucose, D,L-isoproterenol-HCl, quercetin, and Poly-[(GluNa:Tyr)4:1] (PolyGlu$_4$Tyr) were purchased from Sigma Chemical Co. (St Louis, MO). Affinity purified monoclonal antibodies to phosphotyrosine were kindly donated by Mono-Yeda (Rehovot, Israel). Krebs-Ringer-bicarbonate (KRB) buffer, pH 7.4 contained NaCl, 110 mM; $NaHCO_3$, 25 mM; KCl, 5 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 1.3 mM and $MgSO_4$, 1.3 mM. All other chemicals and reagents used in this study were of analytical grade.

Cell preparation and bioassays

Rat adipocytes were prepared from fat pads of male Wistar rats (100–200 g) by collagenase digestion (Rodbell, 1964). Cell preparations showed more than 95% viability by Trypan blue exclusion, at least 3 h after digestion. All bioassays were performed as described in figure legends. *Glucose transport* was carried out using 3-0-[methyl-^{14}C]glucose (Whitesell & Glieman, 1979), *glucose oxidation* was measured by conversion of D-[U-^{14}C]glucose, D-[1-^{14}C] or D-[6-^{14}C]glucose to $^{14}CO_2$ (Rodbell, 1964) and *lipogenesis* by the incorporation of the ^{14}C-labeled glucose analogs into lipids according to Moody *et al.* (1974). *Lipolysis* was evaluated by measuring glycerol released as described previously (Shechter, 1982).

Purification procedures Partially purified insulin receptor was obtained from rat liver membranes as described elsewhere (Meyerovitch *et al.*, 1990). Briefly, crude rat liver membranes were homogenized and the membranal fraction was solubilized with 1% Triton X-100 following centrifugation at 90 000 *g* for 1 h. The supernatant was allowed to pass through a wheat germ agglutinin (WGA)-agarose column and the bound insulin receptor portion was eluted with 0.3 M N-acetyl-D-glucosamine in 50 mM HEPES, pH 7.4, containing 0.1% Triton X-100, 10% glycerol and 0.15 M NaCl. The same procedure was used to obtain solubilized membranes and WGA purified solubilized membranes from rat adipose tissue.

High speed supernatant fractions as a source for CytPTK and PTPase activity were prepared as follows: Fresh adipocytes (for CytPTK activity) were washed three times with KRB buffer, pH 7.4, containing 0.7% BSA, and then twice more with 50 mM HEPES buffer, pH 7.4, containing 1 mM phenylmethanesulfonylfluoride, 10 μg/ml leupeptin and 5 μg/ml aprotinin. The cells (about 10 ml of packed adipocytes) were homogenized with hand teflon homogenizer and then frozen and thawed five times. The fat was removed and the cell homogenate was centrifuged at 40 000 *g* for 60 min. The supernatant was divided into aliquots and stored at −137°C. Alternatively, a cytosolic fraction was prepared from whole adipose tissue homogenized with polytron in 25 mM HEPES, pH 7.5 containing 0.3 M sucrose and a cocktail of protease inhibitors. The supernatant fraction following centrifugation at 100 000 *g* for 1 h was used for PTPase activity.

Tyrosine kinase activity measurement The standard assay mixture (final volume of 60 μl in 50 mM HEPES buffer, pH 7.4–0.1% Triton X-100) contained WGA-agarose-purified InsRTK (0.6 μg protein), 20 mM $MgSO_4$, 1 mM $MnCl_2$, 14 μM ATP and 0.1 μM insulin. Following a 20 min preincubation at 22°C, the reaction was initiated by adding PolyGlu$_4$Tyr (final concentration of 90 μg/ml). It proceeded for 30 min and was terminated by adding EDTA (30 mM). Under these experimental conditions, basel stimulation is low and insulin stimulated PolyGlu$_4$Tyr phosphorylation is ~10-fold higher than the basal. Phosphotyrosine content in PolyGlu$_4$Tyr was quantitated by a radioimmunoassay procedure (Shisheva *et al.*, 1991). This assay included specific monoclonal antibodies to phosphotyrosine (final dilution of 1:100.000) and ^{125}I-BSA-phosphotyrosine conjugate.

For determining CytPTK activity the assay included (in 60 μl of 50 mM HEPES buffer pH 7.4), the enzyme source (0.5–5 μg protein from rat adipocytic high-speed supernatant fraction) 20 mM $MgCl_2$, 2 mM cobalt (II) acetate and 100 μM ATP. Following preincubation (20 min at 22°C), PolyGlu$_4$Tyr (100 μg/ml) was added. The reaction was carried out for 30 min at 22°C, terminated by EDTA and phosphotyrosine content in polyGlu$_4$Tyr was determined as described above.

PTPase activity measurement is estimated by the potency of rat adipocytic cytosol (200 μg protein/ml), rat adipocytic solubilized membranes (200 μg protein/ml) and WGA purified membranes (80 μg protein/ml) to hydrolyze p-nitrophenyl phosphate (pNPP). The assay (50 μl) consisted of pNPP (38 mM in 25 mM HEPES, pH 7.5) the enzyme source and the indicated concentrations of vanadate, tungstate or molybdate. The reaction was stopped by adding 200 μl of 100 mM $NaHCO_3$. The extent of the hydrolysis was determined by spectroscopy at 405 nm following 30 min incubation as described by Kremerskothen & Barnekow (1993).

Protein concentration was determined by the method of Bradford (1976). All the assays were performed either in duplicate or triplicate. Each Figure or Table is the result of a representative experiment performed 3–5 times.

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