Enhanced Sensitivity of Insulin-resistant Adipocytes to Vanadate Is Associated with Oxidative Stress and Decreased Reduction of Vanadate (+5) to Vanadyl (+4)*

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Vanadate (sodium orthovanadate), an inhibitor of phosphotyrosine phosphatases (PTPs), mimics many of the metabolic actions of insulin in vitro and in vivo. The potential of vanadate to stimulate glucose transport independent of the early steps in insulin signaling prompted us to test its effectiveness in an in vitro model of insulin resistance. In primary rat adipocytes cultured for 18 h in the presence of high glucose (15 mM) and insulin (10−7 M), sensitivity to insulin-stimulated glucose transport was decreased. In contrast, there was a paradoxical enhanced sensitivity to vanadate of the insulin-resistant cells (EC50 for control, 325 ± 7.5 μM; EC50 for insulin-resistant, 171 ± 32 μM; p < 0.002). Enhanced sensitivity was also present for vanadate stimulation of insulin receptor kinase activity and autophosphorylation and Akt/protein kinase B Ser-473 phosphorylation consistent with more effective PTP inhibition in the resistant cells. Investigation of this phenomenon revealed that 1) depletion of GSH with buthionine sulfoximine reproduced the enhanced sensitivity to vanadate while preincubation of resistant cells with N-acetylcysteine (NAC) prevented it, 2) intracellular GSH was decreased in resistant cells and normalized by NAC, 3) exposure to high glucose and insulin induced an increase in reactive oxygen species, which was prevented by NAC, 4) EPR (electron paramagnetic resonance) spectroscopy showed a decreased amount of vanadyl (+4) in resistant and buthionine sulfoximine-treated cells, which correlated with decreased GSH and increased vanadate sensitivity, while total vanadium uptake was not altered, and 5) inhibition of recombinant PTP1B in vitro was more sensitive to vanadate (+5) than vanadyl (+4). In conclusion, the paradoxical increased sensitivity to vanadate in hyperglycemia-induced insulin resistant adipocytes is due to oxidative stress and decreased reduction of vanadate (+5) to vanadyl (+4). Thus, sensitivity of PTP inhibition and glucose transport to vanadate is regulated by cellular redox state.

Insulin resistance of glucose transport is a well documented characteristic of type 2 diabetes mellitus (1–3). The etiology of the insulin resistance appears to be multifactorial since it is found in subjects with obesity, those with impaired glucose tolerance, as well as in those with overt type 2 diabetes. There is evidence that both genetic and acquired factors contribute to the insulin resistance (4–6). In type 2 diabetic subjects, peripheral target tissue insulin resistance is characterized by defects in both sensitivity, manifested as a rightward shift in the insulin dose-response curve, and responsiveness, a decrease in maximum response (7–9). This combination of defects has been reproduced in vitro in isolated rat adipocytes incubated for several hours in the presence of a combination of high glucose and high insulin concentrations (10). In this in vitro model, defects in insulin stimulation of glucose transport and glucose transporter translocation are prominent, similar to that found in adipocytes isolated from subjects with type 2 diabetes (9–12).

The signaling pathway by which insulin stimulates glucose transport is only partially understood. Insulin binds to its receptor (IR) and activates its intrinsic Tyr kinase, resulting in phosphorylation of the receptor and its substrates, IRS-1 and -2 (13–15). These, in turn, act as docking proteins for several SH2 domain-containing proteins such as Grb2, SH2 domain-containing phosphatase-2, and p85 (13–16). Binding of the SH2 domains of the p85 subunit of PI 3-kinase results in activation of the p110 catalytic subunit and the generation of the lipid products phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (16, 17). These lipids facilitate phosphoinositide-dependent kinase-1- and -2-mediated phosphorylation of several substrate enzymes such as PKB (Akt/RAC-PK) and atypical protein kinase Cζ and λ (18–21).

The requirement for the IR, IRS 1/2, PI 3-kinase, and either or both PKB and atypical protein kinase Cs for glucose transport stimulation by insulin is well documented, whereas less is known about the more distal signaling events (9, 13–15, 22–24).

Vanadate (+5) and vanadyl (+4) compounds have been well

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documented to mimic many of the actions of insulin (25–28). Glucose uptake and metabolism are stimulated in fat and muscle tissue (29, 30), lipolysis is inhibited (31), and hepatic glucose output is suppressed (32, 33). Oral administration of vanadate and vandyl resulted in lowering of glucose concentrations in rodent models of both type 1 and type 2 diabetes (34–37). Recent studies in human diabetic subjects suggest that these vanadium compounds can lower glycemia and improve lipid levels (38, 39), whereas this treatment was less effective in improving insulin resistance in nondiabetic obese subjects (40).

The mechanism by which vanadate mimics insulin is not completely clear. Although protein-tyrosine phosphatase (PTP) inhibition appears to be important, the role of the IR and/or other Tyr kinases remains controversial (41–43). In some studies, IR kinase activation by vanadate has been documented (44, 45). However, it has been found that insulin-mimetic effects can be stimulated independent of any IR activation (41, 42, 46). The activation of cytosolic (47) and membrane-associated (49) low molecular weight tyrosine kinases have been linked with some of these metabolic effects. However, since these associations were based on inhibition by staurosporine, which inhibits a wide spectrum of kinases, their role remains unclear. In other studies, glucose transport stimulation by vanadate, in contrast to that stimulated by insulin, was found to be independent of the enzymes PI 3-kinase (49, 50) and PKB (50). These data raised the possibility that vanadium compounds may have a unique ability to stimulate and/or enhance insulin metabolic effects in some states of insulin resistance.

To determine the efficacy of vanadate to stimulate glucose transport in an insulin resistant target tissue, we examined its actions in the insulin-resistant rat adipocyte model induced by high glucose and insulin. We found that there was a paradoxical enhanced sensitivity of glucose uptake to vanadate in the insulin-resistant cells, i.e. a leftward shift in the vanadate dose-response curve. This was associated with a concomitant enhanced sensitivity of the insulin-resistant cells to vanadate-stimulated Tyr phosphorylation of the IR and of its intrinsic Tyr kinase activity as well as Ser-473 phosphorylation of the downstream kinase PKB. Investigation of the possible mechanism showed that there was a greater intracellular concentration of vanadate (+5) relative to vanadyl (+4) in the insulin-resistant adipocytes. In vitro studies showed that inhibition of PTP1B phosphatase activity was more sensitive to vanadate (+5) than vanadyl (+4). These data demonstrate that vanadate (+5) is a more potent PTP inhibitor than vanadyl (+4) and that there is decreased reduction of vanadate (+5) to vanadyl (+4) in the insulin-resistant adipocytes. They are also consistent with our observations of increased generation of reactive oxygen species (ROS) in adipocytes exposed to high glucose and insulin and of the influence of altered cellular levels of GSH (glutathione) on vanadate sensitivity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Harlan Sprague-Dawley rats were from Charles River (St. Constant, Quebec, Canada). Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, and fetal bovine serum were from Life Technologies, Inc. Type I collagenase was from Worthington Biochemical Corp. (Freehold, NJ). Human insulin was a gift from Lila Lilly (Indianapolis, IN). 2-deoxy-D-[3H]glucose (10 Ci/mmol) was from PerkinElmer Life Sciences (Mississauga, Ontario, Canada). The GSH assay kit was from Calbiochem (La Jolla, CA). Nitx nylon was from Thompson (Cedarbrook, ON). 5,6-Carboxy-2′,7′-dichlorofluorescein diacetate (DCF-DA) was from Molecular Probes (Eugene, OR). Bovine serum albumin (fraction V), buthionine sulfoximine (BSO), sodium orthovanadate (vanadate), and N-acetylcysteine (NAC) were from Sigma. Vanadyl sulfate was from BD Pharmingen (Mississauga, Ontario, Canada). Anti-Tyr(P) antibody (PY20) was from UBI (Lake Placid, NY) anti-PKB and anti-phosphoserine 473-PKB antibodies were from Cell Signaling Technology (Beverly, MA), and anti-IR antibody was a kind gift from Dr. C. Yip (University of Toronto, Toronto, Ontario, Canada) or from Santa Cruz.

**Preparation of Isolated Adipocytes**—Male Harlan Sprague-Dawley rats weighing 180–200 g were gassed with O2/C02, killed by cervical dislocation, and epidymidal fat pads collected in 3% BSA-DMEM. Adipocytes were isolated as described (41). In some studies, cells were incubated in 3% BSA-DMEM containing 2 mg/ml collagenase for 1 h at 37°C. Cells were then filtered through Nitex nylon (1000 M), centrifuged at 500 rpm for 30 s, and washed twice with 3% and then 1% BSA-DMEM to remove collagenase.

**Primary Culture and Induction of Insulin Resistance**—The isolated adipocytes were preincubated in 1% BSA-DMEM (pH 7.4) supplemented with 0.5% fetal bovine serum and 25 mM HEPES, 1% antibiotics in 250-ml conical culture flasks at 37°C with cells floating on top of the medium in a thin layer. Cells were incubated for 18 h in a humidified atmosphere of 5% CO2 and air. To induce insulin resistance, 10−7 M insulin and 15 mM β-glucose (final concentrations) were added to the medium. Control cells contained no added insulin, and final glucose concentration was 5.6 mM. Cells treated with buthionine sulfoximine (BSO) were incubated in control medium supplemented with 80 μM BSO. Preliminary experiments revealed that this concentration was nontoxic by visual assessment of adipocyte integrity, by trypan blue exclusion, as well as by maintenance of basal and insulin-stimulated glucose uptake similar to that in control cells. The NAC treatment of insulin-resistant adipocytes was carried out with a 2-h pre-exposure of cells to 30 mM NAC at 37°C prior to the induction of insulin resistance for 18 h as described above. After the 18-h incubation, cells were washed twice in 3% BSA-KRBB, pH 7.0 (137 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 1.25 mM CaCl2, 30 mM HEPES, 1 mM sodium pyruvate, and 3% BSA), and then further incubated in the same buffer for an additional 30 min at 37°C to remove any remaining receptor-bound insulin. Cells were then resuspended in 3% BSA-KRBB, pH 7.4 (115 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, 5 mM NaHCO3, 30 mM HEPES, 1 mM sodium pyruvate, and 3% BSA) and washed twice in the same buffer before 2-deoxyglucose (2-DG) uptake assay.

**2-Deoxyglucose Uptake**—The 2-DG uptake assay was performed as described previously (52) with minor modifications. Adipocytes (5 × 106 cells/ml) were preincubated at 37°C with vanadate concentrations from 0 to 2 mM for 60 min. Initial rates of glucose uptake were measured by adding 1 μCi of 2-deoxy-D-[3H]glucose and unlabeled 2-DG (final substrate concentration, 100 μM) to a final volume of 500 μl. At the end of 3 min, the reaction was terminated by adding 500 μl of ice-cold 0.25 mM phlorizin. Nonspecific uptake mediated by simple diffusion and trap-

**IR Autophosphorylation in Situ**—After 18 h of incubation and washing as described above, adipocytes were incubated at 37°C in KRBB in the presence of the indicated concentrations of vanadate for 30 min. The reactions were terminated by immediate freezing to −70°C followed by the addition of ice-cold solubilization buffer (1% Triton X-100, 4 mM EDTA, 2 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 trypsin inhibitor unit/ml of aprotenin, 1 mM vanadate, and 30 mM HEPES, pH 7.6). Adipocytes were homogenized and solubilized for an additional 1 h at 4°C. After removal of the fat cake by centrifugation at 1800 for 10 min, the cell extract was centrifuged further at 100,000 × g for 1 h at 4°C. The supernatant was applied to 1 ml column of WGA-agarose and the IRs eluted with 50 mM HEPES buffer, pH 7.6, containing 150 mM NaCl, 0.1% Triton X-100, and 0.3% N-acetyl-D-glucosamine. 125I-Inulin binding to the lectin purified extract was performed as described previously (53). In some experiments IRs were immunoprecipitated from the solubilized adipocyte extracts with anti-IR antibody.

**Insulin Receptor Tyrosine Kinase Activity**—A procedure similar to that reported previously was followed (45, 53). Lectin-purified IRs (10–20 fmol of insulin binding) were placed in a buffer at 22°C containing 50 mM HEPES, 1 mM MnCl2, 15 mM MgSO4, 2.5 mM mg/poly(Glu-
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Tyr) (4:1), and 25 μM [γ-32P]ATP (5 μCi/tube, pH 7.6) in a total volume of 160 μl. After 10 min, the reaction was terminated by spotting 100 μl of the reaction mixture on filter paper, which was then placed in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. After extensive washing in this solution, the paper was dried and placed in 20 mM Ammonium Hydroxide for determination of radioactivity.

Phosphorylation of PKB—The extent of Ser-473 phosphorylation of PKB was determined as described previously (50). Cell lysates (50 μg) were subjected to SDS-PAGE (10%) followed by electrotransfer to nitrocellulose membranes (Schleicher & Schuell). After blocking for 1 h at 22 °C in Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 and 5% non-fat dry milk, the membranes were incubated overnight with a 1:1000 dilution of either the anti-phospho-PKB antibody or anti-PKB. The membranes were washed and treated for 1 h with secondary antibody (1:10000) conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Proteins were visualized by ECL (Amersham Pharmacia Biotech).

Determination of Cellular GSH—After the 18-h incubation, the different groups of adipocytes were washed and 5 ml of packed cells were resuspended in an equal volume of 5% metaphosphoric acid. After homogenization using a Teflon pestle, the homogenate was centrifuged at 3000 × g for 10 min at 4 °C. The resulting supernatant (100 μl) was used for the GSH assay, which was performed according to the procedure provided by the manufacturer (Calbiochem).

Free radicals were produced and added to the various culture conditions described above. DCF-DA (20 μM final concentration) was added at time 0, and the incubation continued at 37 °C. At the times indicated, a 1-ml aliquot of cell suspension was removed, resuspended in 0.5 ml of PBS, and subjected to flow cytometry analysis (Epics Elite cell sorter, Beckman-Coulter) using an excitation wavelength of 498 nm and detection wavelength of 525 nm (range, 500–545). The mean fluorescence intensity of 10,000 cells from each sample was determined. Signals detected from cells incubated in the absence of probe were considered as background and subtracted. Exposure of adipocytes to H₂O₂ was used as a positive control. Dead cells and debris were excluded by electronic gating of forward and side scatter measurements (54).

Electron Paramagnetic Resonance (EPR) Spectroscopy—After the 18-h incubation, the different groups of cells were washed and incubated with 1 mM adipocytes in 3% BSA-KRBH for 120 min. At the end of the incubation, samples were centrifuged and the infranatant buffer was removed. Three hundred μl of packed cells were pipetted into 500-μl glass tubes, which were flushed with N₂. The tubes were inserted directly into the Bruker TE102 pre-cooled cavity by means of a Teflon sample holder. EPR spectra were collected using a Bruker ECS-106 spectrometer equipped with a model B-VT2000 temperature controller. Liquid N₂ was used to maintain the temperature of the sample, which was measured to an accuracy of ± 0.1 K with a chromium-alumel thermocouple located in the glass Dewar and positioned just below the cavity. The EPR measurements were performed at 223 K.

The EPR instrument parameters were as follows: microwave frequency; microwave power, 25.0 milliwatts; magnetic field, 3365 ± 1000 G; modulation frequency, 50 Hz; modulation amplitude, 8 G. Scanning interval of the magnetic field ranged from 1000 to 1200 G. The EPR spectra parameters were determined by the ECS-106 Bruker data manipulation program. The temperature was chosen to increase the sensitivity and stability of the measurements of rapidly oxidizing paramagnetic centers in biological samples.

Determination of Total Intracellular Vanadium Concentration—After the 18-h incubation, the different groups of cells were washed and incubated for 0–120 min in the presence of 1 mM adipocytes in 3% BSA-KRBH. At the times indicated, the adipocytes were washed five times with PBS made up in double distilled (dd) de-ionized H₂O at 22 °C. In preliminary experiments we determined that there was no vanadium detectable in the fourth or fifth washes after adipocytes were exposed to up to 10 mM vanadate. The cells were separated by centrifugation, and 100-μl aliquots were digested overnight at 23 °C in 100 μl of concentrated (60%) nitric acid. The solubilized supernatant was diluted 50 times in dd de-ionized water. Total vanadium concentrations were determined by atomic absorption spectrometry (55). A standard curve was measured using vanadate dissolved in 0.1% nitric acid made with dd de-ionized water.

Determination of PTP Inhibition—Sodium orthovanadate was dissolved in water and was stored at −20 °C. Vanadyl sulfate was dissolved in water just prior to use to limit oxidation, and N₂ was bubbled through all solutions to prevent oxidation.

Recombinant glutathione S-transferase/PTP-IB (GST/PTP-1B) fusion protein bound to glutathione-agarose beads (Upstate Biotechnol-

ogy Inc., Lake Placid, NY) was washed three times with Buffer A (100 mM HEPES, pH 7.5, 2 mM DTT, and 150 mM NaCl). The GST/PTP-1B fusion protein was then resuspended at a concentration of 1.25 mg/ml in Buffer A supplemented with 125 μg/ml BSA. Five μl of vanadate or vanadyl solution (final assay concentration of 0–100 μM) were placed in the wells of a flat-bottomed, 96-well plate (Corning). Twenty μl of Buffer A was added followed by 20 μl (25 μg) of GST/PTP-1B suspension, and the plate was gently shaken. To begin the reaction, 5 μl of 2 mM 6p600-3m C-terminal phosphoregulatory peptide (TSTFSPqYQF–GENL) or 5 μl of 1 mM IR β-subunit phosphoregulatory peptide (TRDIPYTeDPYpYRK) (Biomol, Plymouth Meeting, PA) were added for a total volume of 50 μl/well. Following a 10-min incubation on a rotator at 22 °C, determined to be in the linear range, the reaction was assayed by adding 100 μl of Biomol Green reagent and the release of P, determined by measuring the absorbance at 630 nm using a Titertek Plus 96-well plate reader. Neither vanadate nor vanadyl (up to 4 mM) interfered with the detection of P.

To determine the effect of DTT on the inhibitors, the above procedure was followed except that Buffer A contained the desired concentration of DTT (0–1.6 mM). Controls were prepared at each concentration of DTT.

Definition of Terms and Statistical Analyses—Results are expressed as percentage above basal. Sensitivity of glucose uptake was determined from the vanadate dose-response curves normalized from basal (0%) to maximum (100%) uptake. IC₅₀ curves were produced using GraphPad Prism 3.0 for Windows (GraphPad Software, San Diego, CA). Quantitative differences among treatment groups were assessed by calculating and comparing the half-maximally effective vanadate concentration (EC₅₀). All values in the text and figures are presented as means ± S.E. All data were subjected to analysis of variance. Probabilities of 0.05 or less were considered to be statistically significant.

RESULTS

Enhanced Sensitivity to Vanadate of Glucose Uptake in Insulin-resistant Adipocytes—The generation of insulin resistance by exposure of freshly isolated rat adipocytes to a medium containing high concentrations of glucose and insulin (high G/I) has been well documented (10, 11). We have determined that this results in a decrease in both responsiveness and sensitivity to insulin. Thus maximum stimulation by insulin after 18-h exposure to high G/I was decreased by 38% (control, 306 ± 20.4 pmol/8 × 10⁶ cells/3 min; resistant cells, 189 ± 24.4; p < 0.01), and the concentration of insulin required to stimulate glucose uptake to 50% of maximum, a measure of sensitivity, was increased in the resistant cells (control, 0.12 ± 0.01 nm; resistant cells, 0.34 ± 0.10 nm; p < 0.05) (data not shown).

In contrast to insulin, when adipocytes cultured in the high G/I medium (resistant cells) were stimulated with vanadate, there was a paradoxical enhanced sensitivity of the resistant cells compared with control. (Note that, throughout these studies, resistant cells refers to adipocytes treated with high G/I and rendered insulin-resistant.) Thus, the vanadate dose-response curve was left-shifted and the concentration of vanadate required for half-maximum stimulation of glucose uptake was 325 ± 7.5 mM in control and 171 ± 92 μM in resistant cells (n = 5, p < 0.002) (Fig. 1). The maximum response to vanadate tended to be lower but was not significantly decreased in the resistant cells (Fig. 1).

Enhanced Sensitivity of the IR to Vanadate in Insulin-resistant Adipocytes—Since the mechanism by which vanadate stimulates glucose uptake appears to involve inhibition of PTPs and support spectral stimulation of Tyr phosphorylation, we examined the ability of vanadate to activate the IR kinase and induce Tyr phosphorylation of the IR. Control and insulin-resistant adipocytes were exposed to vanadate (0–1 mM) for 30 min and IRS1 extracted and partially purified by WGA chromatography. It should be noted that exposure to vanadate concentrations up to 5 mM did not result in greater maximum IR Tyr phosphorylation or kinase activity than observed at 1 mM in either control or resistant adipocytes (data not shown). The insulin-resistant adipocytes showed a paradoxical increased
sensitivity of IR kinase activation by vanadate. The concentration of vanadate required to activate the IR kinase to 50% of maximum was 100 μM in control cells and 26 μM in resistant cells (Fig. 2). The maximum activation of the IR kinase was achieved at 1 mM vanadate and was not significantly different (control, 2.11 ± 0.44 pmol of 32P incorporated/μmol of IR) from that of control medium (1.85 ± 0.44; n = 5) (Table I). We noted that maximum IR kinase activity stimulated by vanadate ranged from 35 to 50% of that achieved by maximum insulin (data not shown).

Activation of the IR kinase is associated with IR Tyr auto-phosphorylation. To determine whether the enhanced sensitivity of the IR kinase to vanadate in the resistant adipocytes was correlated with IR Tyr phosphorylation, the receptors were subjected to immunoblotting with anti-Tyr(P) antibodies. The dose-dependent phosphate incorporation onto Tyr residues of the IR corrected for total IR showed a significantly greater sensitivity to vanadate in the resistant cells (control versus resistant dose-response, p < 0.001) (Fig. 3). The concentration of vanadate that stimulated 50% of maximum IR Tyr phosphorylation was 66 μM in control and 15 μM in resistant cells.

These concentrations were similar to those obtained for activation of the IR kinase above. Maximum Tyr(P) incorporation corrected for IR β-subunit was not different in control and resistant cells (Fig. 3).

Enhanced Sensitivity of PKB to Vanadate in Insulin-resistant Adipocytes—Tyr phosphorylation of the IR and its activation leads to phosphorylation of substrates such as the IRS proteins and subsequent activation of a number of signaling molecules (13, 14). Among these the Ser kinase PKB is prominent and has been implicated in insulin-dependent glucose transport (20–22). Since both IR activation and glucose transport were both more sensitive to vanadate in the insulin-resistant cells, we measured PKB phosphorylation. In the insulin-resistant cells, PKB Ser-473 phosphorylation was more sensitive to vanadate (Fig. 4). PKB Ser-473 phosphorylation has been demonstrated to reflect enzymatic activity in response to insulin (18–20) and vanadium compounds (50).

Enhanced Sensitivity to Vanadate Is Mimicked by BSO and Reversed by NAC—It has been demonstrated that vanadate (+5) is reduced to vanadyl (+4) once it enters cells (56, 57). This appears to be mediated, at least in part, by GSH to which vanadyl can bind (57, 58). However, the oxidized form, vanadate (+5), has been proposed to be more efficacious as a PTP inhibitor than vanadyl (59, 60) and stable complexes of vanadate bound to the active site of PTPs have been successfully crystallized (61). This is consistent with the bipyramidal structure of vanadate, which closely resembles phosphate (61, 62).

We hypothesized that the enhanced sensitivity to vanadate in these insulin-resistant adipocytes may be secondary to a de-
cells with 30 mM NAC for 2 h prevented the fall in GSH concentration (325 ± 18.6 μM, p = not significant compared with control) (Fig. 6).

**Increased ROS Production Induced by High GI**—A decrease in intracellular GSH concentration is an indirect indication of oxidative stress, i.e. the generation of ROS in excess of the antioxidant capacity of the cell. Previous data in other cell types suggest that hyperglycemia can induce oxidative stress and that this is associated with elevated ROS production (reviewed in Refs. 65 and 66). To determine whether the reduction in GSH caused by exposure to high GI was associated with increased production of ROS, the adipocytes were preloaded with DCF and intensity of fluorescence quantified as described under “Experimental Procedures.” DCF fluorescence is a measure of ROS formation and oxidative stress (54, 67). Incubation with high GI increased fluorescence intensity in a time-dependent manner. A significant increase was observed after 3 h of exposure, which peaked by 5 h at 203 ± 23% of control (p < 0.05, n = 4) (Fig. 6). NAC inhibited the increase in fluorescence (106 ± 23% of control). Exposure of adipocytes to 100 mM H2O2, a positive control, augmented fluorescence to 162 ± 11% of control (p < 0.05) (Fig. 7).

**Effect of High GI, BSO, and NAC on the Vanadyl EPR Signal**—As outlined above, intracellular GSH participates in the reduction of vanadate to vanadyl. Thus, it was postulated that in the adipocytes cultured under conditions that resulted in diminished GSH concentration, which was accompanied by enhanced sensitivity of glucose uptake, there would be decreased conversion of vanadate to vanadyl. It is known that vanadyl (+4), but not vanadate (+5), is detected by EPR spectroscopy because it contains an unpaired electron (68). Therefore, the EPR signal intensities reflect the amounts of vanadyl (+4) present in the different cell preparations. After the 18-h culture under the conditions described above, adipocytes were washed and exposed to 1 mM vanadate for 2 h. Equal aliquots of cells were transferred to capillary tubes, rapidly frozen to −50 °C, and subjected to EPR spectroscopy as described under “Experimental Procedures.” BSO treatment as well as exposure to high GI decreased the intensity of the vanadyl EPR signal compared with control (Fig. 8A). This was 39 ± 8% of control in BSO-treated and 47 ± 5% in high GI-treated cells (p < 0.05 for both) (Fig. 8B). Pretreatment of the insulin-resistant (high GI) adipocytes with NAC resulted in a vanadyl signal that was similar to control (114 ± 14%) (Fig. 8).

**Effect of High GI, BSO, and NAC on Total Intracellular Vanadium**—A possible additional explanation for the enhanced sensitivity of insulin-resistant adipocytes to vanadate was that vanadate uptake was augmented in the adipocytes exposed to high GI. Thus, total intracellular vanadium was measured by atomic absorption spectroscopy in equal aliquots of lysates of adipocytes that had been incubated for 60–120 min in 1 mM vanadate under the conditions described above. There were no significant differences in total vanadium after 60, 90, or 120 min between control, high GI-treated, BSO-treated, or NAC-pretreated high GI-treated adipocytes (Table II). Taken together with the EPR data, these results indicate that there was a greater concentration of the more highly oxidized vanadium in the insulin-resistant adipocytes.

**Inhibition by Vanadate and Vanadyl of Recombinant PTP1B**—To determine directly whether vanadate is a more effective PTP inhibitor than vanadyl, the ability of both forms of vanadium to inhibit tyrosine dephosphorylation was assayed using whole adipocyte lysates and recombinant PTP1B. In preliminary experiments it was determined that solutions of vanadate and vanadyl freshly prepared with Na2- treated H2O and buffers remained stable by measuring absorption at 765 nm as

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**FIG. 3. Enhanced sensitivity of insulin-resistant adipocytes to vanadate stimulation of IR phosphorylation.** Rat adipocytes were isolated, cultured overnight, washed, and stimulated with vanadate as described in Fig. 2. Equal amounts of WGA-purified IRS were separated by SDS-PAGE and immunoblotted (IB) with anti-Tyr(P) (pY) and anti-IR β-subunit antibodies. A, representative immunoblot of dose-dependent IR Tyr phosphorylation by vanadate in control and insulin-resistant adipocytes. B, anti-IR β-subunit immunoblot of A. C, normalized vanadate dose-response curves of IR Tyr(P) corrected for IR β-subunit in control (○) and resistant (□) adipocytes. Values are mean ± S.E. of three independent experiments. The two curves were significantly different (p < 0.001, analysis of variance).
described (69). There was no evidence of oxidation of vanadyl to vanadate over at least 45 min (not shown). The solutions were therefore prepared within 30 min of the phosphatase assays, which were of 10-min duration as described under “Experimental Procedures.”

In the PTP1B assay using the pp60c-src C-terminal regulatory peptide as substrate, vanadate (+5) showed a greater efficacy (~6-fold) to inhibit the phosphatase compared with vanadyl (+4) (Fig. 9). Thus, the IC$_{50}$ was 13.6 ± 1.17 nM for vanadate and 83.2 ± 1.15 nM for vanadyl (p < 0.0001). The sensitivity of PTP1B to inhibition using the IR β-subunit phosphoregulatory peptide was found to be less compared with the Src peptide. However, the difference between vanadate and vanadyl was similar with greater inhibition achieved by vanadate at submaximal inhibitory concentrations (Fig. 9, inset). In additional experiments using whole adipocyte lysates and PNPP as substrate, similar results were obtained with vanadate being a more effective inhibitor than vanadyl (percentage of inhibition of dephosphorylation by 1 mM vanadate, 75.3 ± 3.92, and by 1 mM vanadyl, 58.6 ± 9.82) (data not shown).

Since vanadyl can undergo spontaneous oxidation in a neutral pH solution to vanadate, vanadate may be reduced to vanadyl, and both may bind to DTT (69, 70), which was present in the PTP assay buffer, additional experiments were carried out at lower concentrations and without DTT. Recombinant PTP1B remained active at lower and absent DTT. There was a gradual increase in the inhibitory efficacy of both vanadate and vanadyl as DTT concentrations were lowered (data not shown). In the absence of DTT, both inhibited PTP1B activity in the nanomolar range. Under these conditions the relative potency of vanadate was even greater than in the presence of DTT, ~6-fold more potent (IC$_{50}$, 13.6 ± 1.17 nM) than vanadyl (IC$_{50}$, 83.2 ± 1.15 nM, p < 0.0001) (Fig. 10).

**DISCUSSION**

The mechanism of the insulin-mimetic effects of vanadium compounds is not completely understood. Although well documented to inhibit PTPs (25, 31, 71), vanadium compounds have been suggested to directly activate tyrosine kinases (72), inhibit glucose-6-phosphatase (73), inhibit protein degradation (74), alter phosphoinositide metabolism (75, 76), and bind to a variety of small molecules, such as ADP, GDP, and NADH (57, 70), which may all in turn influence cell signaling. However, the predominant effect on PTPs and the ability of general Tyr kinase inhibitors to block the insulin-like actions of vanadium (47–49) indicate that PTP inhibition is the primary mechanism. This concept is also supported by our previous observation that peroxovanadium (pervanadate) is a more potent insulin-mimetic agent and a correspondingly more powerful PTP inhibitor (31).

Whether the more oxidized form vanadate (+5) or reduced form, vanadyl (+4) is the more important and relevant insulin-mimetic form in intact cells and in vivo has been controversial (59, 62, 69). Thus, in some studies it was demonstrated that a cytosolic Tyr kinase was activated by vanadate (+5) while vanadyl (+4) directly inhibited receptor Tyr kinases including the IR kinase (59). In a subsequent report, the same group suggested that vanadyl (+4) had unique properties to activate a cytosolic protein-tyrosine kinase and that adipocytes were more sensitive to vanadyl than vanadate (69). The complex chemistry of vanadium in solution and particularly in living cells (57, 70), along with the uncertainty about which Tyr
kinase(s) is responsible for the insulin-like bioeffects, has made this a difficult question to resolve.

In this study we found that the dose-dependent stimulation of glucose uptake in adipocytes was paradoxically more sensitive to vanadate in cells rendered insulin-resistant by exposure to high glucose and insulin. The increased sensitivity of glucose uptake was paralleled by an increased sensitivity of IR phosphorylation and Tyr kinase activity. Although the role of the IR in mediating the glucose transport stimulated by vanadate is not clear and the IR may not be the kinase involved, its Tyr phosphorylation serves as a cellular marker of PTP inhibition.

Furthermore, we found an enhanced sensitivity of PKB Ser-473 phosphorylation in response to vanadate in the insulin-resistant adipocytes. This Ser kinase has been demonstrated to be a necessary component of the signaling pathway of insulin to stimulate glucose uptake. We noted that the concentrations of vanadate that achieved maximum Ser-473 phosphorylation were higher (~ 5–10 mM) than those required to achieve maximum IR activation (1 mM). Since vanadate is a nonspecific PTP inhibitor, this observation is consistent with activation of other Tyr kinases and recruitment of additional pools of PKB not activated by insulin. Similar results were observed in muscle cells with pervanadate (50). Taken together, these results are consistent with an enhanced activity of vanadate to inhibit PTPs in insulin-resistant cells.

One possible explanation for the enhanced sensitivity to vanadate could have been an elevated PTP activity in the insulin-resistant cells, since the apparent efficacy of vanadium compounds to mimic insulin depends on PTP activity. However, depletion of GSH with diethylmaleate sensitized insulin-resistant cells to vanadate-induced transformation. These results, combined with the proposal that vanadate (77) is the major inhibitor of PTPs rather than vanadyl (79, 80) studies demonstrating that hyperglycemia-induced insulin resistance is not associated with increased IR Tyr dephosphorylation or PTP activity. An alternative explanation was postulated based on several phenomena.

First, it is known that, upon entering cells, vanadyl (+5) is reduced to vanadyl (+4). This may be a mechanism to limit cell toxicity, as vanadate and particularly, pervanadate, have been found to cause oxidative stress (81), DNA damage (82), and induce neoplastic transformation in cultured fibroblasts (83). In the latter study, vanadyl (+4) alone did not induce neoplastic transformation whereas vanadate (+5) had minimal activity. However, depletion of GSH with diethylmaleate sensitized cells to vanadate-induced transformation. These results, combined with the proposal that vanadate (+5) was the major inhibitor of PTPs rather than vanadyl (+4) (see above) suggested that, in our resistant cells, there was decreased reduction of vanadate to vanadyl and/or increased re-oxidation of vanadyl to vanadate. Finally, exposure of various tissues to high concentrations of glucose has been documented to result in oxidative stress (65, 66) and depletion of GSH (84–87).
In the present studies, depletion of GSH with BSO reproduced the leftward shift in the vanadate dose-response curve seen in the insulin-resistant adipocytes. Furthermore, pretreatment of the adipocytes with the antioxidant and GSH precursor, NAC, blocked the enhanced sensitivity caused by the high G/I exposure. To determine whether these perturbations were associated with the predicted changes in vanadate to vanadyl conversion, EPR spectroscopy was performed. This revealed that enhanced vanadate sensitivity was associated with a decreased intensity of the vanadyl (H110014) signal. Since total cell-associated vanadium, determined by atomic absorption, was similar under all these conditions, the EPR results indicate that, in the insulin-resistant and BSO-treated cells, there was a greater amount of vanadate (H110015). In addition, these data imply that the insulin-resistant cells were under oxidative stress. Measurements of GSH showed that, as expected, BSO markedly depleted intracellular GSH. Exposure to high G/I also significantly decreased GSH, which was pre-

**FIG. 7.** Effect of high glucose and insulin on ROS generation in adipocytes. Rat adipocytes were prepared as described and incubated in DMEM containing 5.6 mM glucose (Control), 15 mM glucose and 10^{-7} M insulin (High G/I), 15 mM glucose and 10^{-7} M insulin with NAC (High G/I + NAC), or with 100 mM H2O2 (H2O2). The adipocytes were loaded with 20 μM DCF-DA at time 0. After 1, 3, and 5 h, the cells were washed, resuspended in PBS, and fluorescence intensity measured as described under “Experimental Procedures.” A, representative distribution of log fluorescence intensity of 10,000 cells from one of three experiments with similar results. Rightward shift indicates the presence of increased ROS. B, the mean ± S.E. of the peak fluorescence intensities after 5 h relative to control (100%) are shown (n = 4). *, p < 0.05 compared with control and 15 mM glucose and 10^{-7} M insulin + NAC. The increase in fluorescence intensity induced by 15 mM glucose and 10^{-7} M insulin peaked at 5 h (data not shown) whereas that caused by H2O2 declined gradually from 1 to 5 h.

**FIG. 8.** Electron paramagnetic resonance spectroscopy of vanadate-loaded adipocytes: effect of high glucose/insulin, BSO, and NAC. Rat adipocytes were prepared and cultured for 18 h at 37 °C in DMEM containing 5.6 mM glucose (control), 15 mM glucose and 10^{-7} M insulin (Resistant, R), resistant cells pretreated for 2 h with 30 mM NAC (R+NAC), and control with 80 μM BSO (BSO). The adipocytes were washed and incubated with 1 mM vanadate for 2 h, and aliquots transferred to capillary tubes, which were rapidly cooled to −50 °C and subjected to EPR spectroscopy as described under “Experimental Procedures.” A, the EPR spectra from a representative experiment is shown. B, results shown are the mean ± S.E. relative intensities of spectra determined from three independent experiments (control = 100%). *, p < 0.05 compared with control and resistant cells pretreated for 2 h with 30 mM NAC (see “Results” for details).

**TABLE II**

Vanadium uptake in adipocytes

| Time (min) | Control | Resistant | BSO | Resistant + NAC |
|-----------|---------|-----------|-----|-----------------|
| 60 min    | 16.3 ± 1.3 | 16.1 ± 0.6 | 16.5 ± 0.5 | 16.1 ± 0.3 |
| 90 min    | 21.9 ± 0.9 | 19.8 ± 2.4 | 21.7 ± 1.6 | 21.9 ± 0.6 |
| 120 min   | 31.3 ± 1.2 | 26.11 ± 0.3 | 26.8 ± 0.3 | 26.4 ± 1.4 |

oxidative stress. Measurements of GSH showed that, as expected, BSO markedly depleted intracellular GSH. Exposure to high G/I also significantly decreased GSH, which was pre-
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findings, which demonstrated a greater potency of vanadate compared with vanadyl to inhibit recombinant PTP1B, an intracellular PTP shown recently to be a major regulator of IR Tyr dephosphorylation (88–90). The greater efficacy of vanadate is also consistent with its trigonal bipyramidal structure, which resembles phosphate more closely than the tetrahedral geometry of vanadyl (57, 62).

In a recent study, Cunic et al. (60) reported that cultured Jurkat T-lymphoma cells, which were unresponsive to vanadate, became sensitive to stimulation of protein tyrosine phosphorylation after GSH depletion. Pretreatment of cells with vanadate had no effect on PTP activity of solubilized cell membrane fractions, whereas after GSH depletion ~50% inhibition by vanadate was observed. The authors suggested that the lack of reversibility of PTP inhibition in vitro in the presence of vanadium chelators such as EDTA was consistent with intracellular formation of pervanadate, which was previously demonstrated to cause irreversible inhibition by oxidizing the free PTP sulphydryl to SO2 (91). Since pervanadate, similar to vanadate, is not paramagnetic and not seen by EPR spectroscopy, we cannot rule out a contribution of de novo generated peroxovanadium to the increased sensitivity to vanadate observed in the adipocytes treated with high glucose. However, given that adipocytes are 500–1000-fold more sensitive to pervanadate than vanadate (31, 92) and considering the magnitude of the differences in sensitivity of glucose uptake and PTP inhibition observed, it is not necessary to invoke a contribution of generated pervanadate to explain our results.

In summary, the greater intrinsic potency of vanadate (+5) compared with vanadyl (+4), especially in the nanomolar range as seen in the absence of DTT, along with the higher ratio of vanadate (+5)/vanadyl (+4) concentrations in the insulin-resistant cells appear adequate to explain the shifts in sensitivity of the biological responses seen in the intact cells.

The use of vanadium compounds to treat diabetes has been successful in rodents (25–28). In people with diabetes and overt hyperglycemia, oral administration of vanadium compounds did result in modest improvement in glucose concentrations (38, 39). However, when administered to subjects with insulin resistance who did not manifest hyperglycemia, vanadate had no significant effect (40). These clinical observations are consistent with a more potent effect of vanadium in the presence of elevated glucose. It is possible that the oxidative stress induced by high glucose may increase sensitivity to vanadate in vivo as we found in isolated cells.

Despite the difficulty in achieving effective circulating concentrations of vanadium in humans, interest in the inhibition of PTP1B and concomitant activation of the IR Tyr kinase remains high, particularly since enhanced insulin sensitivity and resistance to high fat diet-induced insulin resistance was reported in the PTP1B (−/−) mouse (90). The specificity of vanadium compounds for different PTPs may be amenable to modification by alteration of ligands (92, 93). This study now demonstrates that the relative potency of vanadium may be regulated by intracellular redox state such that increased efficacy may be achieved in tissues under oxidative stress. This property could be utilized as a method to target PTP inhibition to specific tissues.

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Fig. 9. Inhibition of PTP1B by vanadate (+5) and vanadyl (+4). Recombinant GST-PTP1B phosphatase activity was assayed using the pp60+-C-terminal tyrosine-phosphorylated peptide (TSTEPQpYQPpYK) or the IR β-subunit triple-tyrosine-phosphorylated regulatory peptide (TRDIpYTPypYpYRK) as described under “Experimental Procedures.” The reaction was stopped after 10 min at 22°C by adding Biomol Green reagent and the amount of Pi released quantified by H2P04–/H11006

Values are mean ± S.E. of three to six experiments and for the IR peptide (TRDIpYTPypYpYRK) as described under “Experimental Procedures.” The reaction was stopped after 10 min at 22°C by adding Biomol Green reagent and the amount of Pi released quantified by H23P04–/H11006

Fig. 10. PTP1B phosphatase inhibition by vanadate (+5) and vanadyl (+4) in the absence of DTT. The tyrosine phosphatase activity of recombinant PTP1B was assayed as described in Fig. 9 using the pp60+-C-terminal phosphopeptide as substrate in the presence and absence of 0–1 mM vanadate (+5) or vanadyl (+4) but in the absence of DTT. Values are mean ± S.E. of three to five separate experiments. The sensitivity of PTP inhibition to vanadate (+5) (●) was greater than to vanadyl (+4) (○). The two dose-inhibition curves were significantly different (p = 0.0008), and the IC50 value for vanadate (+5) (13.6 ± 1.17 mM) was significantly lower than for vanadyl (+4) (83.2 ± 1.15 mM, p < 0.0001).

vented by pretreatment with NAC. A decrease in cellular antioxidant concentration is indicative of oxidative stress, which was confirmed by the increased fluorescence intensity of DCFH/DA. Taken together, these experiments indicate that vanadate (+5) is the more potent PTP inhibitor than vanadyl (+4) and that the increased intracellular vanadate (+5) in insulin-resistant adipocytes explains the paradoxical enhanced sensitivity to vanadate. This conclusion, based on results in intact cells, was confirmed by our in vitro...
