Metabolic Effects of Vanadyl Sulfate in Humans With Non–Insulin-Dependent Diabetes Mellitus: In Vivo and In Vitro Studies

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To investigate the efficacy and mechanism of action of vanadium salts as oral hypoglycemic agents, 16 type 2 diabetic patients were studied before and after 6 weeks of vanadyl sulfate ($VOSO_4$) treatment at three doses. Glucose metabolism during a euglycemic insulin clamp did not increase at 75 mg/d, but improved in 3 of 5 subjects receiving 150 mg $VOSO_4$ and 4 of 8 subjects receiving 300 mg $VOSO_4$. Basal hepatic glucose production (HGP) and suppression of HGP by insulin were unchanged at all doses. Fasting glucose and hemoglobin A_{1c} (HbA_{1c}) decreased significantly in the 150- and 300-mg $VOSO_4$ groups. At the highest dose, total cholesterol decreased, associated with a decrease in high-density lipoprotein (HDL). There was no change in systolic, diastolic, or mean arterial blood pressure on 24-hour ambulatory monitors at any dose. There was no apparent correlation between the clinical response and peak serum level of vanadium. The 150- and 300-mg vanadyl doses caused some gastrointestinal intolerance but did not increase tissue oxidative stress as assessed by thiobarbituric acid–reactive substances (TBARS). In muscle obtained during clamp studies prior to vanadium therapy, insulin stimulated the tyrosine phosphorylation of the insulin receptor, insulin receptor substrate-1 (IRS-1), and Shc proteins by 2- to 3-fold, while phosphatidylinositol 3-kinase (PI 3-kinase) activity associated with IRS-1 increased 4.7-fold during insulin stimulation ($P = .02$). Following vanadium, there was a consistent trend for increased basal levels of insulin receptor, Shc, and IRS-1 protein tyrosine phosphorylation and IRS-1–associated PI 3-kinase, but no further increase with insulin. There was no discernible correlation between tyrosine phosphorylation patterns and glucose disposal responses to vanadyl. While glycogen synthase fractional activity increased 1.5-fold following insulin infusion, there was no change in basal or insulin-stimulated activity after vanadyl. There was no increase in the protein phosphatase activity of muscle homogenates to exogenous substrate after vanadyl. Vanadyl sulfate appears safe at these doses for 6 weeks, but at the tolerated doses, it does not dramatically improve insulin sensitivity or glycemic control. Vanadyl modifies proteins in human skeletal muscle involved in early insulin signaling, including basal insulin receptor and substrate tyrosine phosphorylation and activation of PI 3-kinase, and is not additive or synergistic with insulin at these steps. Vanadyl sulfate does not modify the action of insulin to stimulate glycogen synthesis. Since glucose utilization is improved in some patients, vanadyl must also act at other steps of insulin action.

VANADIUM (V) is a natural element that belongs to the first transition series, group Vb (like niobium and tantalum). It is a common constituent of the earth's crust. Similar to elements in group Va (nitrogen, phosphorus, arsenic, antimony, and bismuth), it has 5 valence electrons. Two valence states are most important biologically: tetravalent vanadyl (V^{4+}), usually found as the divalent cation VO^{2+}, and pentavalent vanadate (V^{5+}), VO_3^-.[4,5] Over the past 15 years, considerable evidence has accumulated to show that vanadium salts have insulinomimetic properties in experimental animals, isolated tissues, and cell preparations. Vanadate stimulates hexose transport in rat adipocytes[6,7] and mouse skeletal muscle,[8] stimulates lipogenesis,[9] inhibits lipolysis,[10] stimulates glucose oxidation,[7] and stimulates glycogen synthase in rat adipocytes.[11] In addition to the effects on glucose metabolism, these compounds, like insulin, enhance K^+ uptake in cardiac muscle cells[12] and stimulate DNA synthesis in cultured cells.[13-15]

Oral vanadium salts, vanadyl and vanadate, reduce blood glucose levels in streptozotocin (STZ)-diabetic rats to near-normal values and reverse the catabolic state.[16,17] The beneficial effects are reversible following the removal of vanadium from the drinking water. In the insulin-resistant ob/ob mouse, vanadium salts also reduce glucose levels in the fasting and fed states, improve oral glucose tolerance, and restore early insulin secretion.[18,19] Glucose disappearance rates for intravenous glucose are doubled in vanadium-treated animals as compared with controls. There is also an increase in basal glucose oxidation in muscle in vanadate-treated animals, but not in insulin-stimulated glucose oxidation.[20] Likewise, oral vanadate/vanadyl improves or normalizes blood glucose in obese hyperglycemic db/db mice and in the Zucker fatty rat models of type 2 diabetes.[18,21]

In the late 1800s, vanadium was proposed to have medicinal value and to be of benefit in nutrition, diabetes, atherosclerosis, anemia, metabolism of lipids, prevention of dental caries, and treatment of infection, especially tuberculosis and syphilis.[4,22] Recently, short clinical studies of 2 to 4 weeks' duration by our group and others, administering vanadyl or vanadate at doses of 33 to 50 mg elemental vanadium daily, demonstrate tolerability and improved glycemia with decreased fasting glucose or glycohemoglobin[23-25] and improved insulin sensitivity during euglycemic-hyperinsulinemic clamp studies.[23,26] Some studies have also demonstrated an effect of vanadyl to decrease hepatic

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glucose output during hyperinsulinemia,[23,24] but this has not been observed in other studies.[26] The improvement in insulin-stimulated glucose uptake appears to be mediated primarily through increased nonoxidative glucose disposal[26] and increased glycogen synthesis.[23] The improved insulin sensitivity was sustained 2 weeks after discontinuing vanadyl sulfate.[23] Hyperglycemic clamps demonstrated no change in basal, first-phase, or second-phase insulin or C-peptide secretion.[26] However, a study comparing diabetic and nondiabetic obese subjects on 3 weeks of vanadyl sulfate (50 mg twice daily) found improvements in glucose uptake, glycogen synthesis, and suppression of glucose output only in type 2 diabetic subjects, not in the insulin-resistant obese nondiabetic controls.[27]

To better understand the potential role of vanadium salts in the treatment of type 2 diabetes mellitus, we have administered vanadyl sulfate at doses of 75 to 300 mg daily and evaluated its safety, efficacy, and pharmacokinetics in subjects with type 2 diabetes. We have specifically focused on indices of glycemic control and insulin sensitivity, as well as early insulin signal transduction events, in skeletal muscle.

SUBJECTS AND METHODS

Patients

The experimental protocol was approved by the Human Subject Committee at Brigham and Women's Hospital and Joslin Diabetes Center, and informed consent was obtained from all participants. Sixteen subjects with type 2 diabetes were recruited for the study. Demographic and clinical characteristics of the subjects are shown in Table 1. Eligibility criteria were as follows: age between 18 and 65 years; normal hemoglobin and hematocrit; if female, postmenopausal or surgically sterile; not using nutritional vanadium supplementation; and no history of prosthetic joint replacement (a potential cause of increased serum vanadium levels). In addition, all subjects were free of major active cardiovascular, pulmonary, renal, or hepatic disease.

Protocol

Subjects were studied for 12 weeks. Baseline laboratory testing included glycosylated hemoglobin and fructosamine levels, chest radiograph, electrocardiogram, complete blood cell count, chemistry profile, coagulation times, thyroid function tests, urinalysis, and urine microalbumin. Subjects were instructed to monitor their blood glucose 4 times per day (Lifescan One Touch Meter, Milpitas, CA) prior to meals and at bedtime. At the end of the first study week, subjects were started on placebo 2 tablets orally 3 times daily with meals for a total of 3 weeks. At the end of the placebo treatment interval, laboratory studies were repeated, 24-hour ambulatory blood pressures were recorded, and subjects were admitted to the General Clinical Research Center at Brigham and Women's Hospital. A 2-step euglycemic-hyperinsulinemic clamp study was performed to quantify insulin sensitivity. Deuterated glucose was used to measure endogenous (hepatic) glucose production (HGP), and the study was performed with continuous indirect calorimetry to measure substrate oxidation and energy expenditure.

Table 1. Patient Characteristics

Vanadyl (mg/d)	Age (yr)	Age at Diagnosis	Sex	Weight (kg)	BMI (kg/m²)	HbA_{1c} (%)
75	59	52	M	76.5	26.0	10.7
75	57	57	F	144.1	52.0	12.2
75	57	52	F	105.0	38.6	9.1
150	48	44	M	127.7	37.7	14.2
150	51	44	M	83.1	27.4	9.3
150	65	61	M	79.1	27.1	9.7
150	58	35	M	118.2	39.3	8.5
150	59	49	M	90.9	28.0	11.8
300	54	52	M	163.2	48.7	11.7
300	38	37	M	148.6	37.5	8.4
300	65	63	F	78.2	32.5	8.3
300	51	43	F	70.0	28.8	7.3
300	52	49	M	80.9	27.1	8.6
300	38	30	M	95.2	31.5	17.4
300	43	40	F	61.8	22.0	8.1
300	64	26	M	108.7	37.0	9.8
Mean	53.7 ± 8.3	45.9 ± 10.2		102.0 ± 29.6	33.8 ± 8.1	10.3 ± 2.6

NOTE.

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The subjects were then switched from placebo to vanadyl sulfate 25, 50, or 100 mg orally 3 times daily with meals for 6 weeks (for daily doses of 75, 150, and 300 mg). Blood glucose monitoring, as well as insulin and dietary logs, were continued. A physical examination, blood tests, and urine profiles were repeated every other week throughout the study to test for evidence of toxicity. At the completion of the tenth study week (sixth week of vanadyl administration), outpatient ambulatory blood pressure monitoring and euglycemic insulin clamp studies were repeated and the vanadyl sulfate was discontinued. The subjects were then evaluated for an additional 2 weeks to monitor for adverse effects.

Caloric Intake

Caloric intake was assessed by a dietary history obtained by a trained nutritionist on the first study day to estimate the average daily caloric consumption prior to the study. The subjects were instructed to maintain dietary records for 3 days of each week during the study. Dietary histories were repeated at each visit.

Insulin Sensitivity

To assess insulin sensitivity pretreatment and posttreatment, 2-step euglycemic-hyperinsulinemic clamp studies were performed at 0.5 and 1.0 mU/kg/min.[28] Patients were studied in the postabsorptive state after a 10- to 12-hour overnight fast. Blood glucose was normalized by administration of a low-dose insulin infusion overnight prior to the study. The hand bearing the blood-sampling catheter was placed in a box heated to 70°C to ensure arterialization of venous blood.[29] Catheters were kept patent by a slow infusion of isotonic saline. After collection of baseline samples for hormones and substrates, a primed-continuous infusion of insulin (0.5 mU/kg/min) was administered to increase circulating insulin levels by approximately 300 pmol/L for a period of 120 minutes. At the end of this first insulin infusion period, a second infusion of insulin (1.0 mU/kg/min) was given for an additional 120 minutes to increase plasma insulin levels by approximately 600 pmol/L. Euglycemia was maintained by determining the glucose concentration at 5-minute intervals and adjusting a variable glucose infusion as previously described.[28] Samples for hormone and substrate levels were obtained at intervals throughout the clamp study, including glucose, 6,6-[$^2H_2$]-glucose, and free insulin. Insulin sensitivity is expressed as the metabolic rate (M) of glucose uptake at the two steady-state hyperinsulinemic levels, normalized for the mass (kilograms) of the subject.

Hepatic Glucose Production

The clamp studies were performed with 6,6-[$^2H_2$]-glucose to quantify endogenous glucose production.[30] Beginning 3 hours before the first infusion of insulin and continuing throughout the study, a primed (3.0 mg/kg)-continuous (0.03 mg/kg/min) infusion of 6,6-[$^2H_2$]-glucose was administered. Hepatic glucose production ([HGP] ie, rate of glucose appearance) was calculated by dividing the 6,6-[$^2H_2$]-glucose

infusion rate (in atoms percent excess per minute) by the measured steady-state level of deuterated glucose, in atoms percent excess achieved during the last 20 minutes of the basal state and each step in the hyperinsulinemic clamp.[31] 6,6-[$^2H_2$]-glucose was added to the variable exogenous glucose infusion to maintain a constant level of enrichment during the clamp.[32,33] For the purposes of data analysis, negative rates of glucose production were assumed to represent complete suppression of HGP and were assigned a value of zero.

Oxidative Versus Nonoxidative Glucose Disposal

The clamp studies were performed with continuous indirect calorimetry to assess oxidative versus nonoxidative glucose disposal[34,35] at baseline and during the last 60 minutes of each step of the clamp. Whole-body oxygen consumption, carbon dioxide production, and the respiratory quotient were calculated using the equation of Lusk.[36] A urine sample was collected at the end of the study to measure the nitrogen excretion rate as an index of protein oxidation. From oxygen consumption, CO_2 production, and urinary nitrogen excretion data, carbohydrate, lipid, and protein oxidation rates and energy expenditure were calculated.

Effect of Vanadyl on Insulin-Sensitive Cellular Enzymes

Under sterile conditions with local anesthesia with 1% lidocaine, percutaneous biopsies of the quadriceps muscle were performed before and at the end of the euglycemic-hyperinsulinemic clamp (240 minutes of insulin infusion) both before and after vanadium administration. Biopsy samples were immediately frozen in liquid nitrogen for subsequent assays. Muscle tissue samples with a weight range from 50 to 125 mg were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) at maximum speed for 30 seconds at 4°C in buffer containing 50 mmol/L HEPES (pH 7.5), 137 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 2 mmol/L Na_3VO_4, 10 mmol/L sodium pyrophosphate, 10 mmol/L sodium fluoride, 2 mmol/L EDTA, 1% Nonidet P-40, 10% glycerol, 2 mmol/L PMSF, 10 μg/mL aprotinin, 5 μg/mL leupeptin, and 10 mmol/L benzamidine. Homogenates were allowed to solubilize at 4°C for 30 minutes and were then clarified by centrifugation at 15,000× *g* for 30 minutes.

Immunoprecipitation and immunoblotting. Supernatants of tissue homogenates containing equal amounts of total protein (250 to 750 μg) were immunoprecipitated overnight with anti-insulin receptor CT, anti-IRS-1 CT, or anti-IRS-2 antibodies. Immune complexes were collected with protein A Sepharose (Pharmacia, Piscataway, NJ), washed extensively, and solubilized in Laemmli sample buffer. Proteins were separated using 7.5% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto nitrocellulose (Schleicher and Schuell, Keene, NH), and immunoblotted using ^{125}I-protein A for detection.[37] Quantitation was performed using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Phosphatidylinositol 3-kinase activity. Tissues were homogenized immediately as before and immunoprecipitated with anti-IRS-1-CT antibodies; immune complexes were collected with protein A Sepharose and washed extensively. In vitro kinase assays were performed using phosphatidylinositol (PI) as a substrate.[37] Incorporation of ^{32}P into PI 3-phosphate was quantified using a PhosphorImager.

Glycogen synthase. Muscle homogenates were diluted 1:5 in glycogen synthase assay buffer; synthase activity was measured as previously described.[38] Activity was expressed as the fractional velocity of glycogen synthase, defined as the ratio of activity at 0.1 mmol/L glucose-6-phosphate to that at 10 mmol/L glucose-6-phosphate for each sample.

Phosphotyrosine phosphatase. Approximately 50 mg frozen powdered skeletal muscle from each patient was homogenized in 0.5 mL ice-cold buffer containing 1 mmol/L DTT, 5 mmol/L EDTA, 1 mg/mL aprotinin, 1 mmol/L PMSF, and 250 mmol/L sucrose in 20 mmol/L HEPES, pH 7.4, with 6 up and down strokes of a Polytron (Brinkmann Instruments) at a setting of 4. The crude homogenate was centrifuged at 10,000× *g* for 10 minutes, and the supernatant was centrifuged at 55,000× *g* for 60 minutes. The resulting supernatant was taken as the soluble cytosol fraction, and the pellet was solubilized in 200 μL of the homogenizing buffer containing 1% (vol/vol) Triton X-100 at 4°C for 30 minutes and recentrifuged. Protein was assayed by the method of Bradford.[39]

For use as a phosphotyrosyl protein phosphatase (PTPase) substrate, reduced, carboxamidomethylated, and maleyated (RCM)-lysozyme was tyrosine-phosphorylated.[40] PTPase activity was assayed using 20 μL of the indicated tissue fraction diluted to less than 1 U/mL in buffer and preincubated for 5 minutes at 30°C. The reaction was initiated by the addition of 20 μL phosphotyrosyl RCM-lysozyme (10 mmol/L) and terminated by the addition of 0.9 mL acidic charcoal mixture (0.9 mol/L NaCl, 90 mmol/L sodium pyrophosphate, 2 mmol/L NaH_2PO_4, 4% vol/vol Norit A; Sigma, St Louis, MO). After centrifugation in a microfuge for 1 minute, radioactivity in 0.4 mL supernatant was measured by Cerenkov counting in a liquid scintillation counter. Several time points were taken to calculate an initial reaction rate, which was reported as cpm released per minute per microgram of solubilized membrane protein.

Effect of Vanadyl on Tissue Oxidative Stress

To estimate potential susceptibility to peroxidative changes, serum levels of thiobarbituric acid–reactive substances (TBARS), reflecting lipid peroxidation products such as malondialdehyde and related aldehydes, were determined by mixing a plasma sample (0.2 mL) with 0.67% TBA (2 mL) and 20% trichloroacetic acid (1 mL) followed by incubation at 100°C for 20 minutes. After cooling, the reaction mixture was centrifuged at 3,000 rpm for 5 minutes, and the absorbance of the supernatant was read at 532 nm in triplicate. The concentration of lipid peroxidation products was calculated as malondialdehyde equivalents using the extinction coefficient for the malondialdehyde-TBA complex of $1.56 \times 105\ mol^{-1} \cdot L \cdot cm^{-1}$.[41]

Analytic Methods

Glucose levels were measured by the glucose oxidase method. Free insulin levels were measured as previously described.[42] Deuterated glucose levels were analyzed at Metabolic Solutions (Nashua, NH) by first deproteinizing 200 μL plasma and then preparing an aldonitrile pentaacetate derivative of the glucose in the dried residue using the modified method of Szafranek et al.[43] Measurement of isotopic enrichment was made on a Hewlett Packard (Palo Alto, CA) 5890 Series II gas chromatograph coupled to a Hewlett Packard 5970A mass spectrometer operating in the electron-impact mode. Selective ion monitoring was performed at *m/z* 187 and *m/z* 189 for natural glucose and [6,6-^{2}H]-glucose, respectively. The measured *m/z* 189/*m/z* 187 ratio in glucose was converted to isotope enrichment (mole ratio) with a calibration graph prepared from measured values of standards of known mole ratios. Serum and urine levels of elemental vanadium were determined using a Perkin Elmer (Norwalk, CT) 4110ZL (Zeeman) graphite furnace atomic absorption spectrophotometer.[44] Standard curves were prepared in serum and urine from a commercially available atomic absorption vanadium standard, and quality-control samples were performed every 10 samples. The steady-state period was calculated using the total vanadium present in serum with a first-order kinetic model of the type $C_t = C_0e^{-kt}$, where C_t is the serum concentration at any time (t), C_0 is the initial concentration, and k is the first-order rate constant for elimination of the agent. Twenty-four-hour collections of urine were obtained for analysis of vanadium content during placebo, at days 2, 7, 14, 28, and 42 of treatment, and at days 2, 7, and 14 after treatment discontinuation.

Statistical Analysis

Data are expressed as the mean ± SD unless otherwise noted. Statistical analysis was performed for paired data using 2-tailed Student's *t* tests.

RESULTS

Pharmacology and Toxicity of Vanadyl Administration

Vanadyl was administered 3 times daily with meals over 6 weeks. Compliance was deemed excellent by pill count with greater than 95% adherence. No subject experienced side effects with vanadyl sulfate at 75 mg; however, several subjects experienced gastrointestinal complaints at the 150-mg dose, and all subjects experienced some cramping, abdominal discomfort, and/or diarrhea at the 300-mg dose. At the 300-mg dose, all subjects required treatment with either kaopectate or imodium for these problems. No one withdrew from the study due to these adverse effects. No biochemical evidence of toxicity was detected on the laboratory profiles, which included electrolytes, blood urea nitrogen, creatinine, liver and thyroid function studies, urinalysis, and a complete blood cell count.

Average pretreatment serum vanadium levels were less than 7 ng/mL. There was a 3- to 20-fold range of peak serum vanadium levels at each dose. Peak serum levels and the time to achieve peak serum levels varied greatly between subjects (range, 5 to 44 days of administration). Peak serum vanadium levels were 16.0 ± 5.1 ng/mL (range, 9 to 21), 83.6 ± 44.0 ng/mL (range, 14 to 126), and 284.5 ± 146.3 ng/mL (range, 23 to 513) at 75, 150, and 300 mg vanadyl sulfate, respectively, with a linear correlation between the peak serum level and vanadium dose (R^2 = .993; Fig 1). Peak levels did not correlate with either the apparent bioactivity or side effects. At the 300-mg vanadyl dose, peak serum levels approach the levels achieved in STZ-diabetic rats, which were from 340 to greater than 1,000 ng/mL. Average pretreatment urine vanadium concentrations were less than 5 ng/mL. Peak urine excretion was 0.094 ± 0.05, 0.280 ± 0.25, and 1.21 ± 0.87 mg V/24 h at 75, 150, and 300 mg vanadyl sulfate, respectively. With administration of 300 mg vanadyl sulfate daily, the average amount of vanadium excreted in urine at steady state was approximately 1% of the ingested dose.

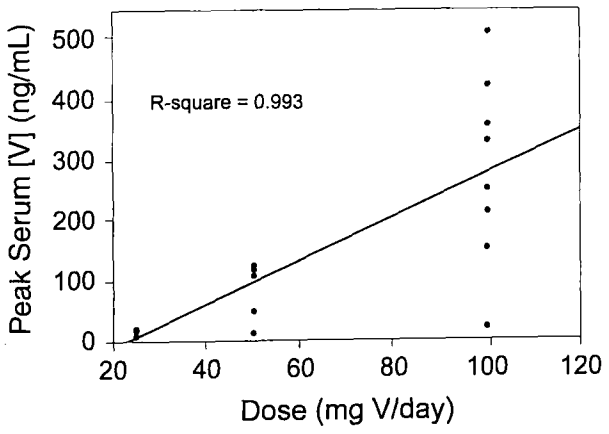

Fig 1. Linear correlation between mean peak serum vanadium concentration (V) and administered dose in subjects with type 2 diabetes.

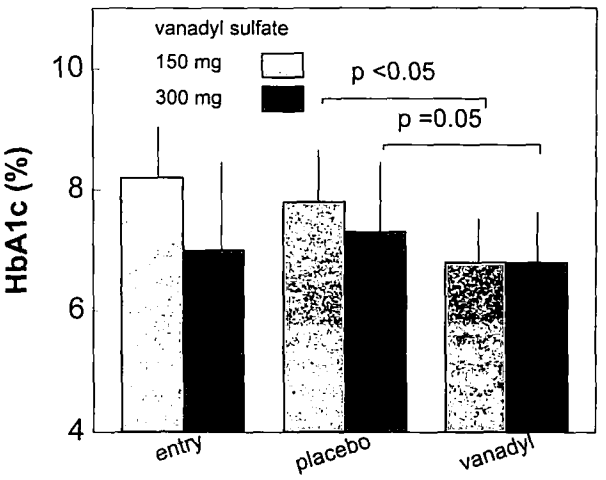

Fig 2. Effect of vanadyl sulfate on HbA_{1c} in type 2 diabetes. There was no significant change in HbA_{1c} in subjects with type 2 diabetes from study entry to completion of placebo. However, there was a decrease in HbA_{1c} after 6 weeks of vanadyl sulfate 150 mg and 300 mg daily, divided.

Diabetic Control

No subjects experienced hypoglycemia requiring assistance during the study. None required a reduction in the dose of their prestudy hypoglycemic regimen, although 1 patient self-discontinued metformin in the middle of the study. There was no significant change in glycohemoglobin or mean fasting glucose in any study group over the placebo treatment period. From the end of the placebo interval to the end of treatment, hemoglobin A_{1c} (HbA_{1c}) decreased significantly in the 150-mg vanadyl group (7.8% ± 1.7% *v* 6.8% ± 1.1%, $P < .05$) and the 300-mg vanadyl group (7.1% ± 2.3% *v* 6.8% ± 2.1%, $P = .05$) (Fig 2). Mean fasting glucose decreased significantly only in the 300-mg vanadyl group (167.2 ± 72.9 *v* 144.1 ± 66.8 mg/dL, $P < .02$). There was no significant change in weight following vanadium treatment in the group as a whole or in the group receiving the highest administered dose of vanadium (104.3 ± 29.4 *v* 103.8 ± 29.4 kg whole group and 107.2 ± 36.5 *v* 106.5 ± 37.0 kg vanadyl sulfate 300 mg daily, pre-V *v* post-V, respectively, P = NS). Furthermore, there was no significant change in caloric consumption by the dietary logs following treatment with vanadyl sulfate at any dose.

Fasting serum cholesterol decreased over the course of treatment in the group receiving 300 mg vanadyl sulfate daily (5.28 ± 0.64 *v* 4.70 ± 0.51 mmol/L, $P < .05$, pretreatment and posttreatment, respectively). This was associated with a decrease in high-density lipoprotein (HDL) cholesterol from 1.02 ± 0.20 to 0.83 ± 0.19 mmol/L ($P < .05$) pretreatment and posttreatment, respectively (Fig 3). There was no significant change in fasting serum triglyceride apolipoprotein A (Apo-A) or Apo-B subfractions (Apo-A 2.47 ± 0.85 *v* 2.34 ± 0.83 and Apo-B 1.98 ± 0.48 *v* 2.01 ± 0.57, P = NS, pre-V and post-V, respectively).

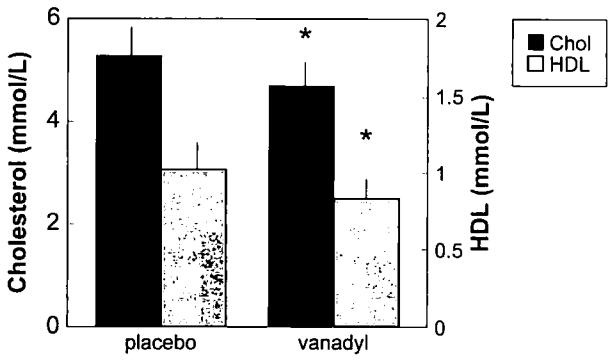

Fig 3. Effect of vanadyl sulfate 300 mg daily on cholesterol in type 2 diabetes. There was a significant decrease in total cholesterol ($P < .05$) following 6 weeks of treatment with vanadyl sulfate 300 mg daily that was not demonstrated at either of the lower doses. However, this was accompanied by a decrease in the HDL cholesterol fragment ($P < .05$).

Effect of Vanadyl on Insulin Sensitivity and Insulin Action

As a primary endpoint of therapy, insulin sensitivity was measured using 2-step euglycemic-hyperinsulinemic clamps at 0.5 mU insulin/kg/min and 1.0 mU insulin/kg/min (Fig 4). Insulin levels achieved during the pre-vanadium (514.1 ± 235.5 and 963.6 ± 369.8 pmol/L, 0.5 and 1.0 mU insulin/kg/min, respectively) and post-vanadium (485.4 ± 205.3 and 946.3 ± 394.9 pmol/L, 0.5 and 1.0 mU insulin/kg/min, respectively) clamps were equivalent. None of the 3 subjects showed an improved rate of glucose utilization after treatment with 75 mg vanadyl sulfate daily; however, insulin sensitivity improved in 3 of 5 subjects when dosed at 150 mg vanadyl sulfate daily, from 30% to 83% and 9% to 230% at 0.5 and 1.0 mU/kg insulin, respectively, and in 4 of 8 subjects when dosed at 300 mg vanadyl sulfate daily, from 47% to 775% and 16% to 75% at 0.5 and 1.0 mU/kg insulin, respectively. However, the effects on the groups were modest and not sufficient to produce a significant change in mean glucose utilization for the group at any of the 3 doses evaluated. No clinical characteristics apparently predicted which subject would respond. There was no apparent correlation between the peak serum vanadium level achieved and the individual change in insulin sensitivity. Basal HGP and suppression of HGP by insulin were unchanged at all 3 vanadium doses, and there was no significant change in oxidative or nonoxidative glucose metabolism.

Effect of Vanadyl Sulfate on Blood Pressure and Tissue Oxidative Stress

Studies in both fructose-fed and spontaneously hypertensive rats suggest a potential antihypertensive effect of vanadyl sulfate.[45,46] To evaluate the potential effect of vanadyl sulfate on blood pressure, subjects wore 24-hour ambulatory blood pressure monitors before and after treatment with vanadyl sulfate.

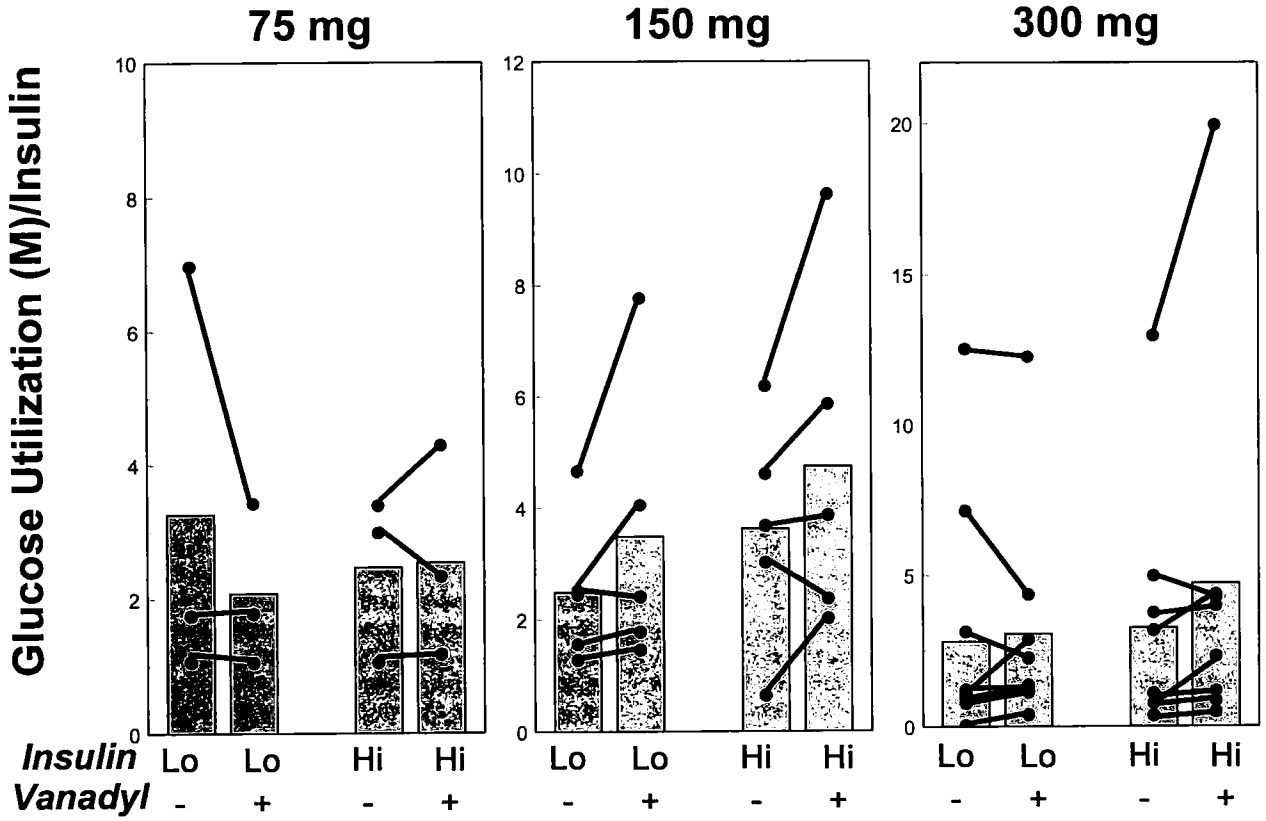

Fig 4. Effect of vanadyl sulfate on glucose metabolism (M)/insulin (M/I) in type 2 diabetes. Insulin sensitivity pre- and post-vanadium treatment was evaluated during 2-step euglycemic-hyperinsulinemic clamp studies at insulin doses of 0.5 mU/kg/min (low dose) and 1.0 mU/kg/min (high dose). Data are shown as glucose utilization (M)/insulin at steady state for each insulin dose. Individual responses are shown as lines, and the mean for the group is shown as the bar. At the middle dose of vanadium, 3 of 5 subjects demonstrated improved insulin sensitivity, and at the higher dose, 4 of 7 subjects demonstrated improved insulin sensitivity.

No differences in systolic, diastolic, or mean arterial pressure or heart rate were demonstrated at any of the 3 vanadyl doses administered.

Rodent studies suggest that vanadium salts may increase tissue oxidant stress. To estimate the potential susceptibility to peroxidative changes, the effect of vanadyl sulfate on lipid peroxidation was assessed in plasma pre- and post-vanadium treatment. Lipid peroxidation products, TBARS, were unchanged by vanadyl sulfate 300 mg daily (3.51 ± 0.50 v 3.68 ± 0.43 μmol/L, P = NS).

Molecular Mechanisms of Insulin Action

Percutaneous biopsies of the quadriceps muscle were performed before and at the end of the 2-step hyperinsulinemic clamps (240 minutes of insulin infusion) before and after 6 weeks of vanadyl treatment. Given the important role of PI 3-kinase–dependent pathways in insulin-stimulated glucose disposal,[47-51] IRS-1–associated PI 3-kinase was measured in muscle homogenates and found to increase 4.7-fold following insulin infusion in the pre-vanadyl clamp (P = .02). Following vanadyl treatment, basal levels of IRS-1–associated PI 3-kinase were increased by 4.2-fold over the control ($P < .01$); however, there was no further increase in PI 3-kinase activity by insulin (Fig 5). Similarly, insulin stimulated the tyrosine phosphorylation of the insulin receptor, Shc, and IRS-1 proteins (2.7-, 1.9-, and 3.2-fold, respectively) in the pre-vanadyl state; following vanadyl treatment, there was a consistent trend for increased basal levels of insulin receptor, Shc, and IRS-1 protein tyrosine phosphorylation, but no further increase with insulin (Fig 6).

Since previous human studies with sodium metavanadate demonstrated that improved insulin sensitivity was largely mediated through increased nonoxidative glucose disposal,[26] we assessed the activation of glycogen synthase in muscle homogenates. Glycogen synthase fractional activity increased 1.5-fold, with a mean increase in fractional velocity of 109% ± 46% following insulin infusion, but there was no change in either basal or insulin-stimulated activity post-vanadium at the vanadyl sulfate 150-mg dose.

Phosphatase activity was evaluated in muscle homogenates. There was no association between PTPase levels and responsiveness to vanadyl. There was a suggestion that vanadium increased PTPase in the tissue particulate fraction, although this

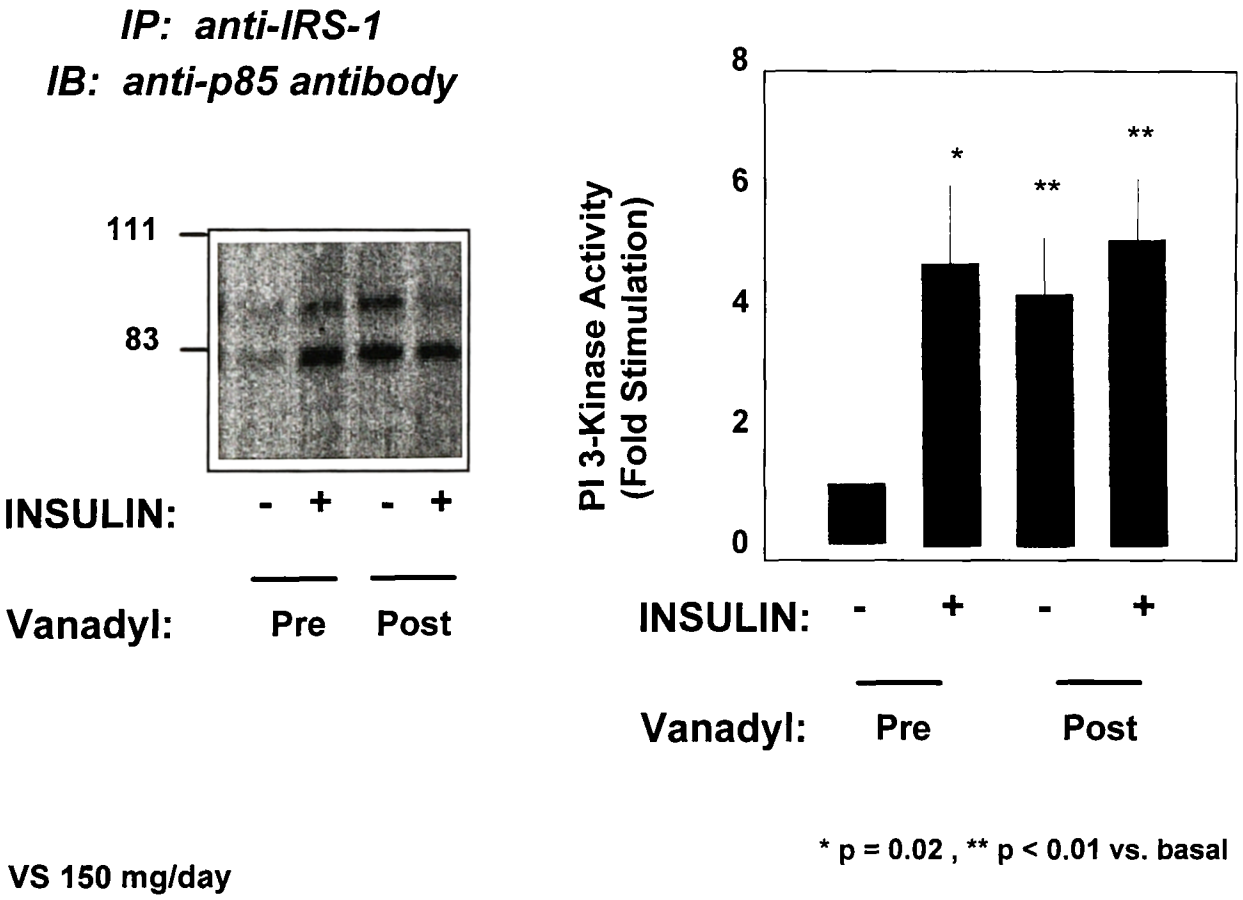

Fig 5. IRS-1 association with p85 regulatory subunit of PI 3-kinase and activation of kinase activity. To evaluate potential molecular mechanisms of action of vanadium salts, IRS-1–associated PI 3-kinase was measured in muscle homogenates before and at the end of the 2-step euglycemic clamp both pre- and post-vanadium treatment, and was found to increase 4.7-fold following insulin infusion in the pre-V clamp (P = .02). Following V treatment, basal levels of IRS-1–associated PI 3-kinase were increased by 4.2-fold over control ($P < .01$); however, there was no further increase by insulin. IRS-1 association with the p85 regulatory subunit of PI 3-kinase is shown after immunoblot with anti-p85 antibody and immunoprecipitation with anti-IRS-1 antibody. (Left) In vitro kinase assay was performed using phosphatidylinositol as a substrate (right).

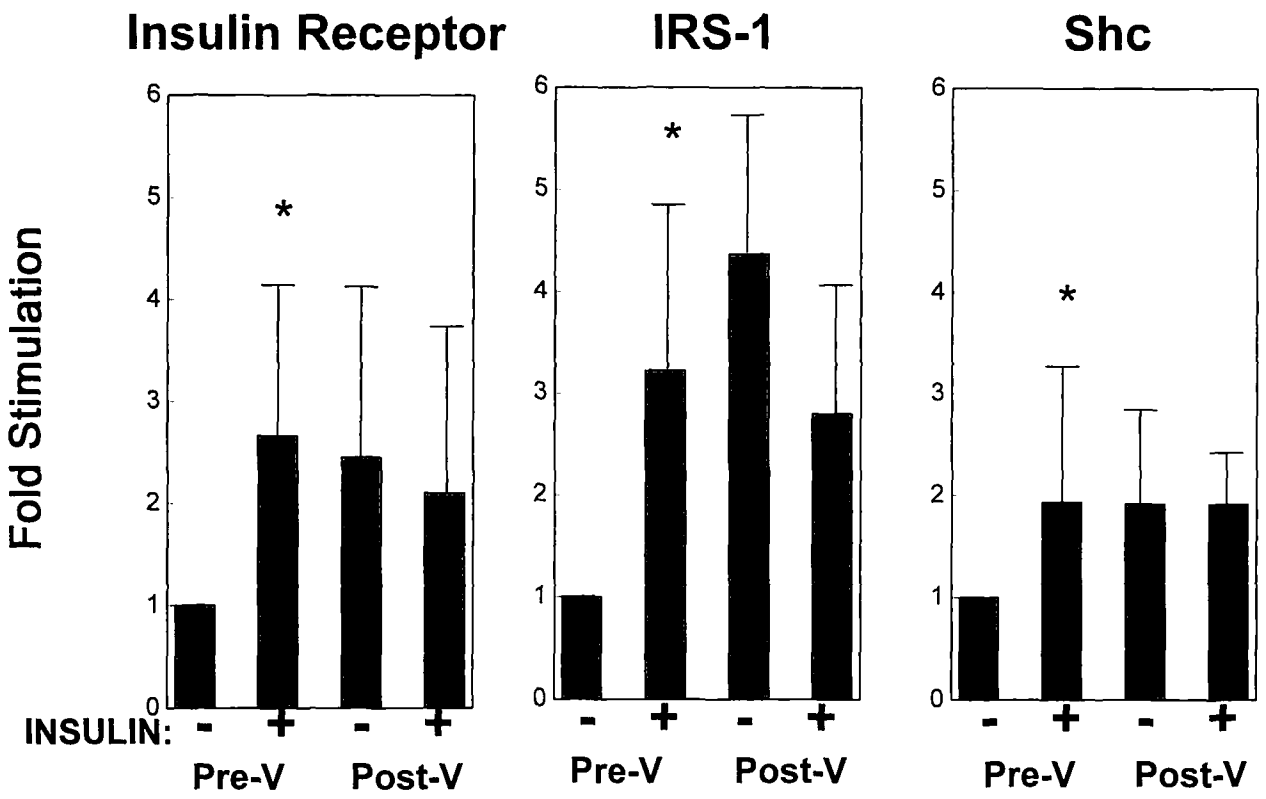

Fig 6. Protein phosphorylation in human muscle before and after vanadyl sulfate. Insulin stimulated tyrosine phosphorylation of the insulin receptor, IRS-1, and Shc proteins (2.7-, 3.2-, and 1.9-fold, respectively) in the basal state. Following 150 mg vanadyl treatment, there was a consistent trend for increased basal levels of insulin receptor, Shc, and IRS-1 protein tyrosine phosphorylation, but no further increase with insulin.

did not reach statistical significance, due to a large standard deviation.

There was no discernible correlation between tyrosine phosphorylation patterns, glycogen synthase activity, or PTPase levels and either the patient response to vanadyl or the peak serum vanadium level.

DISCUSSION

Two oxidative states of vanadium, vanadyl and vanadate, have been shown to have insulin-mimetic properties in a number of isolated cell systems[6-11] and in animal models of both type 1 and type 2 diabetes.[18,19,21,52-54] The few clinical trials in humans have been less consistent. Following 2 weeks of treatment with sodium metavanadate (125 mg/d), we found that insulin sensitivity improved in patients with type 2 diabetes mellitus, as well as some patients with type 1 diabetes.[26] Increased insulin sensitivity was primarily due to an increase in nonoxidative glucose disposal, whereas oxidative glucose disposal was unchanged. Both basal and insulin-mediated suppression of HGP were unchanged, although it was difficult to rule out an effect on hepatic insulin sensitivity, since HGP was maximally suppressed even by low-dose insulin. Likewise, Cohen et al[23] demonstrated improved insulin sensitivity in clamp studies after 3 weeks of vanadyl sulfate (100 mg/d) in 6 subjects with type 2 diabetes mellitus, with a near doubling of the glucose infusion rate required to maintain euglycemia. This was associated with no change in basal HGP but increased insulin suppression of HGP. Oxidative glucose metabolism was increased after treatment. The metabolic improvements persisted for at least 2 weeks after discontinuation of the study agent. However, other human trials have failed to demonstrate improvements in insulin sensitivity and/or carbohydrate oxidation in type 2 subjects[24] or obese nondiabetic subjects,[27] and demonstrated no change in either the insulin requirement or insulin sensitivity in subjects with type 1 diabetes.[55] The lack of consistent improvement in clamp measurements of insulin sensitivity in these human trials could be due to low subject numbers (5 to 10 subjects) in each trial, recruitment bias, or differences in the dose, preparation, timing of administration, or form of vanadium salt administered. Although insulin-mimetic properties can be demonstrated with both vanadyl and vanadate, these salts may have different pharmacokinetics, bioavailability, or intracellular effects. The current investigation included subjects treated with diet, sulfonylureas, and/or metformin, whereas in previous studies subjects were treated with sulfonylurea or diet alone, and vanadium salts may have positive effects when used alone or in combination with sulfonylureas, but these effects may not be additive to or synergistic with biguanides.

In general, smaller effects have been observed in humans as compared with studies in rodents, which may reflect differences in the administered dose, attained blood or appropriate tissue levels, and time course of action. For example, in rodents, the dose of oral vanadate that has been found to improve blood glucose is about 100 mg/kg/d, while the dose used in the current

human study is about 1.5 mg/kg/d. Likewise, the blood level of vanadium achieved in rodent studies is between 10 and 20 μmol/L, similar to the effective vanadium concentrations necessary to inhibit phosphatases in in vitro studies. Peak serum levels in the present human trial were only 1 to 10 μmol/L. Finally, in humans, the longest treatment period remains 6 weeks. While vanadate added to the drinking water reduces glucose to near-normal values within 3 to 4 days in STZ-diabetic animals,[16] the effect of vanadate to decrease blood glucose to near-normal levels in ob/ob and db/db mice requires 10 to 20 days.[18] Thus, it remains possible that studies of longer duration in humans may be necessary to fully evaluate the potential of this compound in glucose metabolism. Gastrointestinal complaints are likely to limit further dose increases until more potent and tolerable forms of vanadium are developed, although it is possible that starting with lower doses and escalating slowly may be better tolerated.

The current study design of a placebo lead-in trial has some limitations, as improvements found during the treatment phase may be solely due to participation in a research trial. However, this effect is minimized by the extended placebo interval before baseline evaluation. Furthermore, there is evidence that vanadium salts have persistent effects after administration of the compound is discontinued,[56,57] thus making a placebo crossover design invalid. If improvements in glycemia and insulin sensitivity were demonstrated, a parallel placebo trial would be necessary. However, as the trial was essentially negative and patients could not tolerate the product without side effects, this design is not currently warranted.

Large variation was observed in the achieved serum vanadium levels at the two higher doses. Lower levels could not be directly explained by either compliance as assessed by pill count or severity of gastrointestinal side effects. It is possible that the timing of administration with respect to meals or the meal content varied among subjects. Absorption may differ between individuals, or gastrointestinal side effects may affect absorption. Since no direct correlation was found between the peak serum level and the magnitude of glycemic response, it is possible that serum levels do not clearly reflect the levels achieved at the cellular site of action.

Vanadate has previously been studied in humans for its potential in the treatment of hypercholesterolemia.[58] Vanadium salts may inhibit cholesterol synthesis by interference with the formation and utilization of mevalonic acid,[59] and inhibit coenzyme A and thus interfere with the conversion of HMG to β-methyl crotonate.[60] Deposition of cholesterol is reduced and mobilization of predeposited cholesterol is increased in rabbits fed vanadium,[61,62] and in healthy human subjects administered oxytartratovanadate, small reductions in total and free cholesterol levels and small increases in serum triglycerides were demonstrated.[58] Previous human trials demonstrated a significant reduction in serum cholesterol in both type 1 and type 2 diabetic patients with sodium metavanadate[26] or vanadyl sulfate[25] treatment, although these results are not found in all studies.[23] It is possible that the cholesterol reduction is dose-related, as we demonstrated this only at the highest dose administered. Reduced cholesterol was not accompanied by a significant change in fasting serum triglyceride Apo-A or Apo-B subfractions. However, it is of concern that the decrease in cholesterol was associated with a decrease in the HDL cholesterol fraction.

Essential hypertension is associated with multiple metabolic defects in carbohydrate and lipoprotein metabolism, which include insulin resistance, hyperinsulinemia, and dyslipidemia.[63,64] Animal models of insulin resistance, hyperinsulinemia, and hypertension include the spontaneously hypertensive rat (SHR),[65] a genetically transmitted model of hypertension, and the fructose-fed rat,[66] an acquired, diet-induced model of systolic hypertension. Vanadyl sulfate has been shown to reduce plasma insulin and systolic blood pressure without affecting plasma glucose in both the SHR and fructose-fed rats.[45,46] When insulin was administered subcutaneously to restore plasma insulin to pretreatment levels, the effects of vanadyl to reduce blood pressure were reversed. In addition, chronic oral administration of bis(maltoloto)oxovanadium (IV), an organic vanadium complex that has also been demonstrated to reduce plasma insulin concentrations in nondiabetic rats without affecting glucose levels,[67] can cause a sustained reduction in plasma insulin and systolic blood pressure and improve insulin sensitivity as measured by euglycemic clamp in the SHR.[68] These studies did not measure the serum vanadium levels associated with the blood pressure–lowering effects, but the administered doses were similar to those used to decrease glucose in diabetic rodents. Even at the highest dose of vanadyl sulfate administered in the current study, there was no effect on mean systolic or diastolic pressure, mean arterial pressure, or heart rate as assessed by ambulatory monitoring after 6 weeks.

Oster et al[69] suggest the potential for vanadate to increase oxidative tissue damage, demonstrating a trend for increased TBARS in vanadium-treated animals. This is of clinical concern due to the potential link between oxidative damage and diabetic complications. However, there was no change in any measure of the antioxidant defense system including the activities of liver or kidney Se-dependent and non–Se-dependent glutathione peroxidase, glutathione reductase, CuZn-superoxide dismutase (SOD) and Mn-SOD, or oxidized and reduced glutathione concentrations. Thompson and McNeill[70] also evaluated the effects of vanadyl feeding on oxidative stress and found that the treatment was antioxidative with respect to cataract formation and reduced glutathione concentrations in liver homogenates, pro-oxidative by iron-stimulated TBARS assays, and inconclusive with respect to glutamine synthesis activity. Thus, the results can vary depending on the tissue and the assay, with liver and kidney effects having potential adverse risk in humans. However, the interpretation of results with regard to the long-term risk of diabetic complications remains difficult. To evaluate potential susceptibility to peroxidative changes, we measured serum levels of the lipid peroxidation product, TBARS, as these markers were consistently abnormal in rodent studies. We found no evidence of tissue oxidative stress, as reflected by TBARS, with vanadyl sulfate at doses up to 300 mg daily.

The molecular mechanism of the vanadium effect on insulin signaling remains uncertain, and several potential sites for the insulin-like effect have been proposed. Insulin action at the cellular level is complex.[71,72] In vitro vanadate stimulates an 8-fold increase in 2-deoxyglucose uptake in trypsin-treated adipocytes.[73] This treatment removes most of the α-subunit of

the insulin receptor including the ligand binding site, and indicates that vanadate stimulates glucose transport via some mechanism other than binding to the insulin receptor. Tamura et al[1] have presented evidence that vanadate might directly stimulate insulin receptor β-subunit tyrosine autophosphorylation; however, this action has not been observed in several other studies.[2,3] Furthermore, the effect of vanadate on hexose transport is not inhibited by quercetin, a compound that inhibits both insulin receptor tyrosine kinase and insulin-stimulated hexose uptake,[74] implicating an alternate mechanism. Vanadium may stimulate a soluble cytosolic tyrosine kinase, thus bypassing the need for activation of the insulin receptor itself.[75] In contrast to vanadium salts, peroxides of vanadium appear to produce activation of the insulin receptor tyrosine kinase as measured by ^{32}P incorporation into a synthetic substrate, and in some studies they are even more potent than insulin itself.[76,77]

Other candidate enzymes for vanadium action are downstream in the insulin signaling pathway. Insulin activates a series of closely linked serine/threonine kinases, such as mitogen-activated protein (MAP) kinase, and phosphatases, including protein phosphatase-1A, important for activation of glycogen synthesis. Like insulin, vanadate administration has been shown to increase both S6 phosphorylation and S6 activation in rat liver[78,79] and in skeletal muscle of STZ-diabetic rodents.[80] Furthermore, activation of MAP kinases does not appear to require insulin receptor phosphorylation.[81] Similarly, elevations in basal phosphorylation levels of both MAP and ribosomal S6 kinases were demonstrated in circulating mononuclear cells of diabetic subjects treated with sodium metavanadate.[26] In an attempt to evaluate proteins involved in insulin signaling upstream of major metabolic pathways in an important insulin-sensitive tissue, we evaluated insulin receptor, IRS-1, and Shc protein tyrosine phosphorylation and IRS-1–associated PI 3-kinase activity in a skeletal muscle biopsy before and after treatment. Following vanadium therapy, there was an increase in basal tyrosine phosphorylation, but no further increase following insulin infusion. However, since increased glucose utilization was demonstrated by insulin infusion during clamp studies in some subjects, other pathways must also be involved in the metabolic effects of vanadyl sulfate.

Vanadate is a potent inhibitor of PTPases,[82,83] and much attention has focused on the possibility that vanadate stimulates phosphorylation of the insulin receptor either directly or via its inhibitory effect on PTPases. Vanadate has been shown to preferentially inhibit particulate, or membrane-associated, PTPase relative to cytosolic PTPase in hepatoma cells.[84] However, at vanadyl sulfate doses of 300 mg daily, inhibition of phosphatase activity to exogenous substrate in muscle homogenates did not appear to account for the increase in the signaling protein phosphorylation status. These data suggest that overall tissue PTPases may not be an accurate reflection of the tissue target for vanadyl to enhance insulin signaling, which could involve interactions between the vanadium and specific phosphatase enzymes that are not measured here and are difficult to study in isolation. Since the clinical response to vanadyl sulfate does not correlate with the overall muscle tissue PTPase activity, it remains possible that either the activity of specific PTPases is affected by vanadium compounds, PTPases in specific tissues are differentially affected, or an inhibition which occurs in vivo is lost in vitro.

In vitro, vanadium salts stimulate glycogen synthase in rat adipocytes.[1,11] In previous human trials in vivo, glycogen synthase, assessed by isotopic glucose incorporation into glycogen, was increased following vanadyl administration.[23] However, despite increased tyrosine phosphorylation of insulin receptor, IRS-1, PI 3-kinase, and Shc proteins, there was no change in basal or insulin-stimulated glycogen synthase fractional activity after vanadyl administration. Different results in the analysis of glycogen synthesis could be due to different assay techniques or the failure of this study cohort to demonstrate improved insulin sensitivity or carbohydrate oxidation overall, whereas the group evaluated by Cohen et al[23] did demonstrate improved sensitivity. However, the regulation of phosphorylation of the upstream signaling proteins has been thought to be instrumental in the regulation of glycogen synthase, and the discordance in upstream phosphorylation and glycogen synthase activity will require further investigation.

The long-term safety of vanadyl sulfate administration at pharmacological doses could not be assessed within the context of this study; however, vanadyl appears safe and relatively well tolerated at doses of 75 to 300 mg daily for 6 weeks. At these doses, insulin sensitivity or glycemic control does not dramatically improve in all individuals. Vanadyl sulfate modifies early steps in insulin signaling in human skeletal muscle, including basal insulin receptor and substrate tyrosine phosphorylation and activation of PI 3-kinase, but is not additive or synergistic with insulin at these steps. Vanadyl sulfate does not modify the action of insulin to stimulate glycogen synthesis. Since glucose utilization is improved in some patients, vanadyl must also act at other steps of insulin action. Further studies are warranted to evaluate the discordance between early and later steps of insulin signaling. Safe and more potent analogs of vanadium will be necessary before vanadium therapy can be used therapeutically in human diabetes mellitus.

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