Gluconeogenesis in Non-Obese Diabetic (NOD) Mice: In Vivo Effects of Vanadate Treatment on Hepatic Glucose-6-Phosphatase and Phosphoenolpyruvate Carboxykinase

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The contribution of gluconeogenesis to hyperglycemia in non-obese diabetic (NOD) mice has been investigated using oral vanadate administration. Vanadate compounds have been shown to mimic many actions of insulin; however, the exact mechanism is poorly understood. The aims of the present study were (1) to elucidate vanadate's action in vivo, and to assess the possibility that its glucose-reducing effect is dependent on the presence of a minimal concentration of insulin; and (2) to evaluate the effects of vanadate administration on the key hepatic gluconeogenesis enzymes, glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK), as well as glucose-6-phosphate dehydrogenase (G-6-PDH). Vanadate caused a significant reduction in blood glucose but failed to normalize it, despite effective serum vanadate concentrations (26.2 ± 1.6 μmol/L). Two weeks after initiation of treatment, blood glucose levels were 26.0 ± 1.8, 21.7 ± 3.0, 16.0 ± 1.6, and 14.3 ± 2.3 mmol/L in the control (C), insulin (I), vanadate (V), and combined vanadate and insulin (V + I) groups, respectively ($P < .001$). G-6-Pase activity was significantly reduced by vanadate (622 ± 134 *v* 365 ± 83 nmol/min/mg protein in C *v* V, $P < .05$). PEPCK activity was also significantly reduced (844 ± 370, 623 ± 36, 337 ± 43, and 317 ± 75 nmol/min/mg in the C, I, V, and V + I groups, respectively, $P < .001$). No significant differences in the hepatic glycogen stores and G-6-PDH activity were noted between treatment groups. Our study suggests that the inhibition of hepatic G-6-Pase and PEPCK activity by vanadate plays an important role in reducing blood glucose levels in NOD mice.

INSULIN-DEPENDENT diabetes mellitus (IDDM) is an autoimmune disease that involves the destruction of insulin-producing cells in the islets of Langerhans. Despite current treatment protocols, the long-term microvascular and macrovascular complications of IDDM and the evolution of insulin resistance continue to stimulate the investigation of new treatment strategies.

Vanadate, a trace element, has been shown to be a potent insulinomimetic agent in isolated adipocytes[1] and in streptozotocin (STZ) diabetic rats,[2-4] rodent models of non–insulin-dependent diabetes mellitus (NIDDM),[5] cats,[6] and obese fa/fa rats.[7] Recent studies evaluated its effectiveness in the treatment of diabetes in humans with NIDDM.[8,9] Despite intensive research, vanadate's mechanism of action is not clearly understood.[9]

The non-obese diabetic (NOD) mouse spontaneously develops autoimmune diabetes that mimics human IDDM, and it is therefore regarded as an excellent animal model of human IDDM.[10] The immunologic pathophysiology of IDDM has been extensively investigated, but the effect of vanadate administration in NOD mice has not been characterized. The aims of the present study were thus to characterize vanadate's actions in NOD mice, especially its effect on gluconeogenesis, to assess the combined in vivo effect of vanadate with minimal concentrations of insulin, and to evaluate vanadate's action on the key gluconeogenic hepatic enzymes, glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK).

MATERIALS AND METHODS

Animals

Ninety-nine female NOD mice aged 8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed in groups of 5 in polypropylene cages. All mice were maintained according to the *Guidelines for the Care and Use of Laboratory Animals* (US Department of Health and Human Services, 1996) inside a barriered rodent facility. Prediabetic mice received water in plastic bottles containing 80 mmol/L NaCl. The mice were acclimatized for 10 days before experimentation, during which period the health status of the animals was assessed daily by observation. Before commencement of the study, all mice were examined and weighed and the blood glucose level was measured using the Glucometer Elite (Bayer Diagnostics, Puteaux, France).

Experimental Design

Blood glucose concentrations were measured once weekly by sampling a drop of blood from the tail. Mice with blood glucose of 11.1 mmol/L or higher were regarded as diabetic, and allocated randomly to 4 groups according to treatment schedule as follows: (1) Control (C) mice received no treatment. The drinking water contained 80 mmol/L NaCl. (2) Insulin-treated (I) mice were injected subcutaneously with 1 IU insulin NPH (Humulin-N; Lilly, Fegersheim, France) every other day. This treatment has been shown in preliminary studies to be anabolic but inadequate to normalize blood glucose levels. The dose of insulin was adjusted to cause a slight decrease in blood glucose and maintain body weight. These mice also received 80 mmol/L NaCl in the drinking water. (3) Vanadate-treated (V) mice received sodium metavanadate (BDH Chemicals, Poole, UK) in the drinking water at a concentration of 3.92 mmol/L. Vanadate solutions were replaced every 3 days. (4) Vanadate and insulin (V + I) mice received sodium vanadate in the drinking water at a concentration of 3.92 mmol/L and were injected subcutaneously with 1 IU insulin NPH every other day. Vanadate solutions were also replaced every third day.

All animals were inspected daily. Each mouse was weighed every other day, and blood glucose was measured before insulin was injected. Water consumption was measured 3 times weekly. Seven or 14 days

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after initiation of treatment, mice were anesthetized by ether inhalation, exsanguinated, and killed. The blood was separated, and the sera were stored at −20°C. A full necropsy was performed on each mouse. Liver samples were immediately frozen in liquid nitrogen and stored at −70°C.

Serum Vanadate Assay

Terminal blood samples were analyzed for vanadate levels using a Perkin-Elmer flameless atomic absorption spectrophotometer (5100 ZL Zeeman Furnace Module; Perkin-Elmer, Norwalk, CT). The sensitivity of the assay was 0.2 μmol/L vanadate. Each sample was assayed in duplicate, from which a mean was derived.

Glycogen Determination

A piece of frozen liver was digested by 33% KOH, and the glycogen was precipitated with 66% ethanol and determined as described by Hujing.[11]

Enzyme Assays

Frozen liver was homogenized in a 10-mL vol of 0.25-mol/L sucrose containing 10 mmol/L Tris, pH 7.4, and 0.5 mmol/L EDTA (STE buffer). The microsomal fraction was isolated according to the method of Arion et al.[12] Briefly, the tissues were prepared from 10% liver homogenate in 0.25 mol/L sucrose, 10 mmol/L HEPES, pH 7.4.[12] The homogenates were centrifuged for 10 minutes at 8,000 × *g*, and the resultant supernatants were recentrifuged for 45 minutes at 105,000 × *g*. The obtained microsomal fraction was used for determination of G-6-Pase activity, and the supernatants were used for determination of PEPCK and glucose-6-phosphate dehydrogenase (G-6-PDH) activities.

G-6-Pase activity was measured in the microsomal fraction according to the method of Burchell et al[13] with slight modification. The microsomal fraction was diluted to 2 mg protein/mL and incubated for 10 minutes at 5°C with 0.2% deoxycholate (DOC). Since the isolated microsomes were a mixture of intact and disrupted vesicles, preincubation with DOC disrupted most of the microsomal vesicles. G-6-Pase activity was assayed at 30°C in a final vol of 0.2 mL containing 50 mmol/L Tris/cacodylate buffer, pH 6.5, 15 mmol/L albumin, 20 mmol/L glucose-6-phosphate, and 25 μg DOC-treated microsomal protein. After a 10-minute incubation, the assay was terminated as previously described.[13] This resulted in an approximately 2-fold increase in enzyme activity. The activity of G-6-Pase in the isolated microsomes was studied in the presence of 1 mmol/L mannose-6-phosphate according to the method of Arion et al,[12] and thus the total catalytic activity was determined.

G-6-PDH activity was present in the cytosolic fraction of the homogenate as described by Bergmeyer.[14] Protein content was determined as previously described.[15] PEPCK activity was determined according to the method of Chang and Lane[16] using the rate of exchange between $KH^{14}CO_3$ and unlabeled oxaloacetate.

Data Analysis

Data are presented as the mean ± SEM. The unpaired Student's *t* test (2-tailed) was used to compare 2 groups, and ANOVA was used to compare multiple groups.

RESULTS

Blood Glucose

Blood glucose levels are presented in Fig 1. Blood glucose in untreated C mice continued to increase throughout the study period. Two weeks after initiation of the treatments, blood glucose levels were significantly lower in V and V + I groups. The maximal effect of vanadate was achieved 24 to 48 hours after initiation of treatment, and thereafter a constant mild elevation was the rule; however, blood glucose did not reach normoglycemic values (5 ± 1.6 mmol/L). A highly significant negative correlation was found between blood glucose and serum vanadate concentrations for V and V + I mice ($P < .002$; Fig 2).

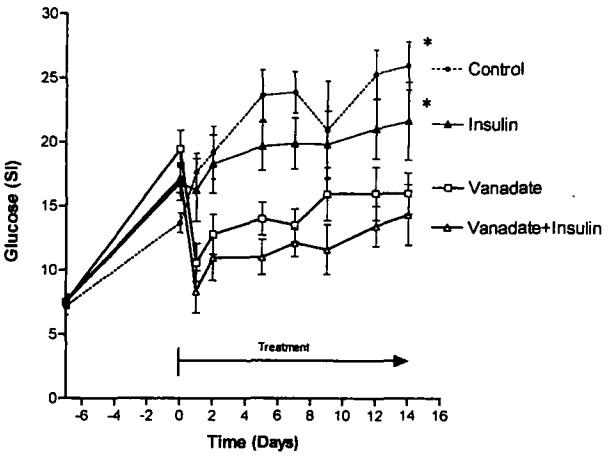

Fig 1. Blood glucose in the 4 treatment groups. Blood glucose concentrations were measured once weekly by sampling a drop of blood from the tail. *$P < .02$.

Body Weight

Vanadate treatment initially caused a slight decrease in body weight, reaching a maximal effect in the first 24 to 48 hours after the start of treatment. However, 2 weeks after the initiation of treatment, no significant differences in body weight change were noted between C and V groups.

Serum Vanadate

Serum vanadate concentrations were similar at both 7 and 14 days after initiation of treatment. Seven days after treatment started, mean vanadate concentrations were 26.2 ± 1.6 μmol/L (range, 12.6 to 41.6).

Hepatic G-6-Pase Activity

Vanadate caused a significance decrease in hepatic G-6-Pase activity 7 days after initiation of treatment (Fig 3). The mean hepatic G-6-Pase activity was 622 ± 54, 587 ± 89, 364 ± 34, and 369 ± 39 nmol/min/mg for the C, I, V, and V + I groups, respectively ($P < .05$). A positive linear correlation was found between the terminal blood glucose levels and G-6-Pase activity ($P < .0001$; Fig 4).

Hepatic PEPCK Activity

Seven days after treatment initiation, hepatic PEPCK activity was significantly reduced in the V treatment group compared with the C group (844 ± 186 *v* 337 ± 21 nmol/min/mg for C *v* V, $P < .05$; Fig 5). PEPCK activity was suppressed to a greater extent than G-6-Pase in the V groups (6% *v* 40%).

Hepatic Glycogen Stores

Vanadate administration did not cause significant changes in the hepatic glycogen content 7 days after treatment initiation.

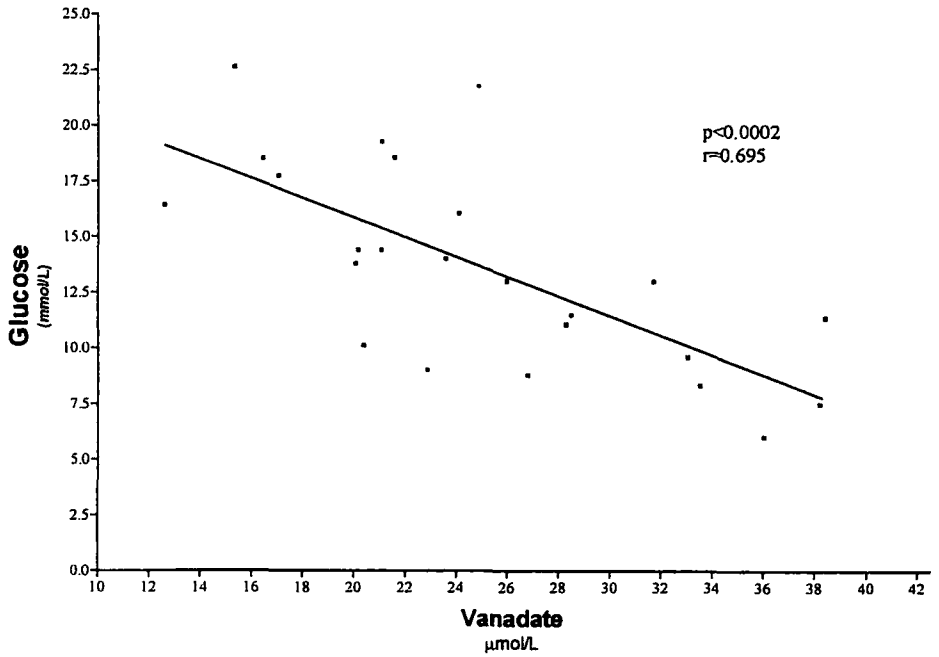

Fig 2. Correlation between final blood glucose and serum vanadate levels. Terminal blood samples were analyzed for vanadate using the Perkin-Elmer flameless atomic absorption spectrophotometer.

Hepatic G-6-PDH Activity

Vanadate treatment tended to cause an increase in hepatic G-6-PDH activity hinting at an enhancement of lipogenesis; however, no statistical significance was reached. Hepatic G-6-PDH activity in the study groups was 8.25 ± 0.34, 10.18 ± 1.17, 11.98 ± 1.31, and 11.77 ± 1.42 nmol/min/mg for C, I, V, and V + I treatment groups, respectively. A significant negative correlation was found between the terminal blood glucose level and G-6-PDH activity ($P = .02$) (Fig 4).

DISCUSSION

In the present study, we present the first characterization of the various metabolic effects of oral vanadate treatment in hyperglycemic NOD mice. Vanadate administration for 2 weeks significantly decreased but did not normalize blood glucose levels, despite effective vanadate serum levels. We previously reported that similar blood levels of vanadate normalized blood glucose in STZ diabetic rats and ob/ob mice,[4,6] as well as the nutritionally induced diabetic *Psammomys obesus italicus.*[17]

The dissimilar response of diabetic NOD mice compared with other species with similar protocols of vanadate treatment has several possible explanations. An altered sensitivity of the putative target protein(s), eg, cytosolic tyrosine kinase, mitogen-activated protein and S6 kinase, and tyrosine phosphatase,[9] between different animal species may explain these discrepancies. However, since we observed beneficial effects of vanadate administration in 2 other mouse models,[5] this explanation is unlikely. Another possibility is that vanadate effects in vivo are

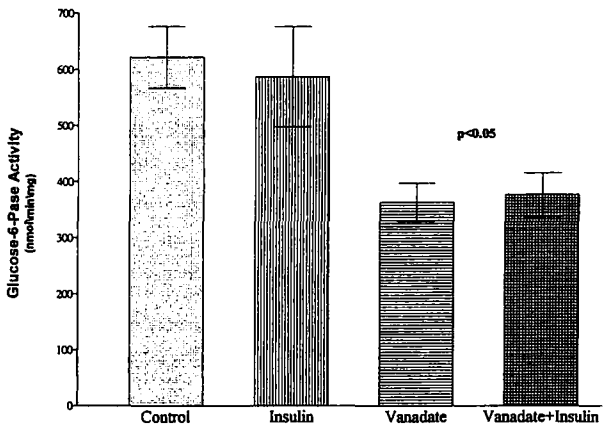

Fig 3. Effect of treatment with V, I, and V + I on hepatic G-6-Pase activity 7 days after treatment initiation. Frozen liver was homogenized and the cytosolic fraction was isolated. G-6-Pase activity was measured in the cytosolic fraction according to Burchell et al.[13]

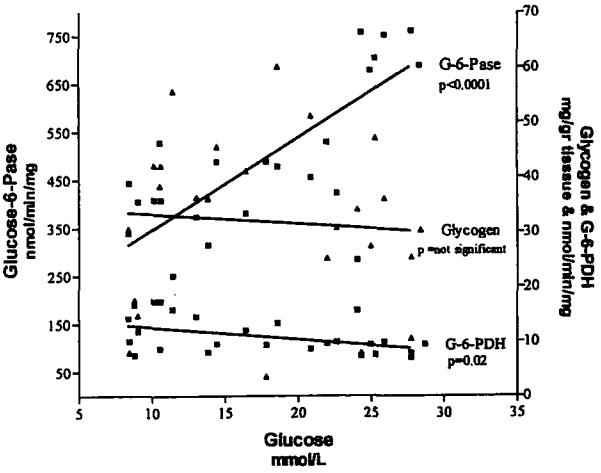

Fig 4. Correlation between final blood glucose level and hepatic G-6-Pase and G-6-PDH activities and glycogen level 7 days after treatment initiation. Frozen liver was homogenized, the cytosolic fraction was isolated, and G-6-Pase and G-6-PDH activities and the glycogen level were determined.

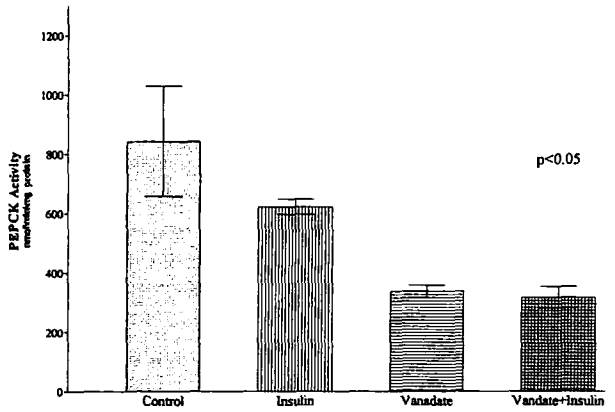

Fig 5. Effect of treatment with V, I, and V + I on hepatic PEPCK activity 7 days after treatment initiation. Frozen liver was homogenized, the cytosolic fraction was isolated, and PEPCK activity was determined[16] by the rate of exchange between $KH^{14}CO_3$ and unlabeled oxaloacetate.

less prominent in an insulin-deficient state such as exists in NOD mice in comparison to rodents with elevated insulin levels. This possibility was assessed by treatment of the NOD mice with anabolic doses of insulin, which were effective in inducing small but significant reductions of blood glucose and increases of body weight. However, the results of our study show that the V + I combination did not cause a synergistic effect on blood glucose, and even this combination treatment did not normalize blood glucose levels.

Serum vanadate levels in our study were about 20 μmol/L. This vanadate concentration range has been shown to induce 5% to 15% of the maximal biological response in isolated cells.[18] In human studies, the range for vanadate concentrations was between 1 and 5 μmol/L. Vanadate therefore appears to have greater beneficial metabolic effects in vivo compared with isolated cell systems. The reason for this may be an increase of specific intracellular concentrations of vanadate during prolonged exposure.[19] Another reason may be the enhancement of insulin binding to its receptor.[20] Previous studies suggested that vanadate's in vivo effect may be distal to the tyrosine phosphorylation of the insulin receptor,[21] and it may reduce blood glucose levels by affecting several sites, such as increasing peripheral glucose uptake.[3] The decrease in blood glucose may reduce expression of the gene for the catalytic unit of G-6-Pase.[22,23]

We report for the first time that vanadate treatment inhibits in vivo hepatic G-6-Pase and PEPCK activity, and blood glucose levels in NOD mice correlate with the activity of these enzymes. The combination of V + I treatment was found to have no additional effect on G-6-Pase and PEPCK, suggesting a common regulatory pathway for the effects of vanadate and insulin. These results suggest that the hypoglycemic effects of vanadate may be mediated also via suppression of these important enzymatic steps in liver glucose production.

In vitro studies by Singh et al[24] demonstrated the inhibition of G-6-Pase by micromolar concentrations of vanadate (50% inhibitory concentration [IC_{50}], 7 μmol/L). Sekar et al[25] reported that in fat cells, vanadate inhibited in vitro G-6-Pase at similar concentrations. In our study, serum vanadate levels were about 26.2 ± 1.6 μmol/L. These vanadate concentrations are sufficient to inhibit G-6-Pase activity, based on the previous studies by Singh and Sekar et al, although we did not prove it by directly determining the intracellular glucose-6-phosphate content. Thus, vanadate may cause reduced G-6-Pase activity at the level of both gene expression and inhibition of enzyme activity.

These findings support the hypothesis that the inhibition of gluconeogenic enzymes is independent of other metabolic and enzymatic alterations induced by vanadate in the diabetic state, eg, enhancement of peripheral glucose uptake. In humans and fa/fa rats,[26] the primary metabolic effect of vanadate was not due to decreased hepatic glucose production, but rather to increased peripheral glucose disposal. Both the insulin-deficient state and the higher vanadate concentration in our study may explain the main effect of vanadate to suppress gluconeogenesis. Glucose utilization was not assessed in this study, but it was also probably improved, based on previous studies.[8,9,26]

The effect of vanadate in the human was studied using small doses and yielded clinical effects including some insulin sensitivity and a decrease in blood glucose levels.[9] The combination of these results with ours suggests a possible beneficial effect of combined V + I treatment in IDDM. However, the use of vanadium compounds is probably more efficient in NIDDM states because vanadium acts distally to the locus of insulin resistance, reducing the daily requirement for insulin and thus preventing the vicious circle of increasing insulin dosage, increasing body weight, and increasing insulin resistance. Future studies on more potent vanadate derivatives are suggested to improve its clinical use in insulin-resistant states.

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