EFFECTS OF METAVANADATE ON THE LIVER GLUCOSE-6-PHOSPHATASE IN BB RAT

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Abstract. Diabetes-prone BioBreeding (DP-BB) rat develops a spontaneous disorder that closely resembles human insulin-dependent (type I) diabetes mellitus but the initial events that cause the diabetes onset remain largely unknown. Administration of metavanadate, an insulin mimetic agent, has been shown to normalize the glucose-6-phosphate level in liver and to decrease hyperglycemia in DP-BB rat. The purpose of this study was to investigate the effects of vanadium administered in the drinking water to BB rat on the hydrolytic and phosphotransferasic activities of hepatic microsomal G6Pase. This treatment decreased the both activities, proportionally with dose applied, the phosphotransferase activity to a greater extent. Elevated level of carbamyl phosphate: glucose phosphotransferase activity after 83 days from the treatment ceasing may explain the success obtained in postponing diabetes onset.

Key words: type I diabetes, plasma glucose levels, BB rat, sodium metavanadate, rat liver, glucose-6-phosphatase, phosphohydrolase and phosphotransferase activities.

INTRODUCTION

Vanadium compounds have been shown to have insulin-mimetic activity, both *in vitro* and *in vivo* [14, 27]. Their antidiabetic effects have been demonstrated in streptozotocin diabetic rats, in other animal models, such as spontaneously diabetic (BB) rats, insulin-resistant Zucker *fa/fa* rats and recently in human trials [4, 9, 10, 12, 13]. In most of these studies, vanadium compound was given orally in the drinking water. There are many unanswered questions with regard to how vanadium salts mimic the actions of insulin [5, 25]. Vanadate interacts potently with phosphatases and the inhibition is attributed to a five-coordinate vanadate complex, which mimics the transition state of the phosphate ester hydrolysis reaction. It has been found to be a potent inhibitor of both the hydrolytic and synthetic activity of glucose 6-phosphatase (G6Pase) system. The inhibition by vanadate is competitive with the phosphate substrate and is greater for the

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phosphotransferase than the hydrolase activity of the system [26]. Schultz [24] considers that the suppression of G6Pase in diabetes by vanadate administration may contribute to the normalizing effect of its on blood glucose.

Liver plays a major role in the regulation of blood glucose levels. In response to stress or low blood glucose levels, it releases glucose for use by other tissues. The terminal step of both glycogenolysis and gluconeogenesis, the two glucose producing pathways, is catalyzed by glucose 6-phosphatase (G6Pase), that is a multicomponent, integral membrane enzyme system located in the endoplasmic reticulum (ER). According to the substrate-transport model, the G6Pase system comprises a catalytic subunit and putative accessory transport proteins for glucose-6-phosphate, inorganic phosphate and glucose [2, 11]. G6Pase is capable not only of catalysis of the hydrolysis of glucose-6-phosphate and inorganic pyrophosphate but of glucose-6-phosphate synthesis via a phosphotransferase activity [21] from glucose and a variety of phosphoryl donors as carbamyl phosphate and pyrophosphate.

The amount of G6Pase can be increased or decreased under physiopathological and/or nutritional conditions associated to increased or decreased hepatic glucose production, respectively [1]. The rate of hydrolysis of glucose-6-phosphate by hepatic G6Pase system and the hepatic output of glucose were increased in all the diabetic conditions studied [3, 6, 18]. The prolonged insulin deficiency and hyperglycemia cause a marked increase in the hepatic G6Pase mRNA and protein and indicate that short-term (~8 h) correction of hyperglycemia in diabetic rats leads to normalization of the hepatic gene expression of this enzyme, regardless of the circulating insulin level [17]. Recent studies have suggested that increased G6Pase and/or decreased glucokinase may explain the increase in endogenous glucose production in type 2 diabetic patients [8].

The aim of our experiments was to investigate effects of sodium metavanadate on the hydrolytic and phosphotransferasic activities of liver multifunctional Glc6P*ase* in BB rats.

ANIMALS AND EXPERIMENTAL DESIGN

BB rats were obtained from Dr. P. Thibert, Animal Resources Division, Health Protection Branch, Health & Welfare Canada, Ottawa, Canada.

96 BB-DP rats of prediabetogenic age (aged 40 – 50 days) were divided into four groups: N, V_1,V_2 and V_3 (n = 24 each), homogeneous as age (mean aged 40 days), weight (about 150 g) and sex. In all, 40 – 60% of diabetic × diabetic sibling crosses of the BB strain of rats become diabetic with the onset beginning at about 60 days of age and peaking at about 100 days of age. The control group (N) received, for 7 days, a drinking solutions of 0.5% NaCl in water. The three test groups received, for 7 days, a drinking solution consisting of NaCl 0.5% and one of

the following sodium metavanadate solution: 0.1% (V_1), 0.2% (V_2) and 0.7% (V_3). After this treatment, all animals were received standard diet *ad libitum* and tap water (without NaCl, $NaV0_3$) and monitored over a period of 90 days for changes in weight, glucosuria and glycemia to detect onset of diabetes.

MATERIALS AND METHODS

Every day during the study, tail vein blood was collected from animals for determination of plasma glucose levels, using a Glucometer One-Touch.

Three normal animals from each group (C, V_1, V_2 and V_3) were sacrificed after 7, 45 and 90 days of experiment. All diabetic animals were anesthetized and killed. The liver was quickly perfused with cold 0.15 M NaCl, removed and homogenized in 0.25 M sucrose, 10 mM Hepes, pH 7.4 (9 ml/g fresh mass). After centrifugation at 10 000 × g for 10 min at 4 ºC, the supernatant was recovered and centrifuged at 100 000 × g for 1 h at 4 ºC. The 100 000 × g pellet was washed once under the latter conditions and was suspended in the homogenization buffer (0.9 ml/g wet liver). The term detergent-treated microsomes refers to microsomes that were further solubilized in the presence of 0.5% (mass/vol.) sodium cholate for 20 min at 4 ºC before experiments.

The hydrolase and phosphotransferase activities of Glc6Pase system were studied in detergent-treated rat liver microsomes.

Glucose 6-phosphohydrolase activity of the system was measured by the method of Burchell *et al.* [7] at 37 ºC for 10 min in a buffer (pH 6.5) containing 50 mmol/l sodium cacodylate, 2.0 mmol/l EDTA and the following glucose-6-phosphate concentrations: 1.7, 2.5, 3.3, 5.0, 10 or 15 mmol/l in a total volume of 200 µl. The assay was initiated by addition of 20 µl of the microsomal preparation containing ~ 10 µg protein. After 10 min, the reaction was stopped by the addition of 0.8 ml of stopping reagent (3.4 mmol/l ammonium molybdate in 0.5 mol/l sulfuric acid, 0.52 mol/l sodium dodecyl sulfate and 0.6 mol/l ascorbic acid in the proportion of 6:2:1, made fresh daily). The color was developed by incubation at 45 ^{o}C for 20 min, and absorbance was determined at 820 nm on a Beckman spectrophotometer. The hydrolase activity was expressed in µmol/min/mg protein.

Phosphotransferase activity of G6Pase system was measured as described by Nordlie and Arion [19] with carbamyl phosphate, the most effective phosphoryl donor substrate yet demonstrated. Assay mixtures (pH 5.5) contained, in 1.5 ml, 50 mmol/l sodium cacodylate; 40, 50, 70, 100 or 200 mmol/l glucose; 1.0, 2.0, 3.0, 5.0, 10 or 20 mmol/l carbamyl phosphate; and cholate-treated microsomes (0.14 mg protein). The glucose-6-phosphate formed was measured enzymatically with glucose-6-phosphate dehydrogenase and $NADP^+$. A reference molar absorbance index of 6.22×10^3 $M^{-1}cm^{-1}$ is utilized in calculating glucose-6-phosphate concentrations. The transferase activity was expressed in nmol/min/mg protein.

Protein concentration was assayed by the method of Lowry [16] using bovine serum albumin as a standard.

Statistical analysis was performed according to Student's *t*-test. Differences were considered significant when the *p* value was less than 0.05.

Results

In the first day after cessation of the oral treatment with vanadate (V_1, V_2 and V_3 groups) and with 0.5% NaCl (C group), three normal rats from each experimental group were sacrificed, and the microsomes have been separated from the liver. We determined the kinetic parameters for G6Pase (hydrolase and transferase activities) of the detergent-treated microsomes.

The K_M and V_{max} values for the hydrolase activity of the G6Pase system were calculated from the Lineweaver and Burk plots. In control group, the kinetic parameters of the hydrolase activity were found to be 0.96 ± 0.09 mM for K_M and 0.99 ± 0.1 μmol/min/mg microsomal protein for V_{max} (Fig. 1).

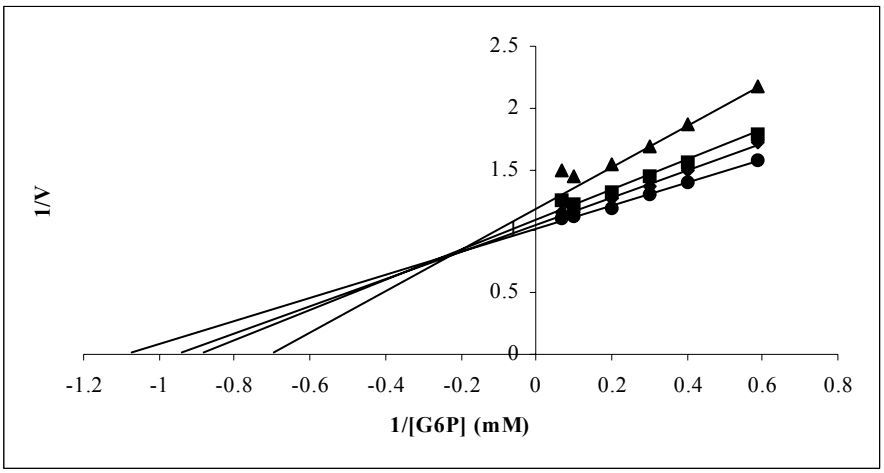

Fig. 1. – The Lineweaver-Burk plots for microsomal glucose 6-phosphohydrolase from BB rats belonging to groups C (●), V_1 (♦), V_2 (■) and V_3 (▲).

In the same manner, we performed kinetic studies on the hydrolase activity from normal and diabetic rats belonging to the C, V_2 and V_3 groups on days 45 and 90 of the experiment (Table 1).

During the monitoring study, in the normal BB rats (C group), no statistically significant differences were found between the K_M values, while the maximal velocities for the hydrolase activity of the G6Pase system increased with the rat ageing. In diabetic and untreated BB rats (C group), the kinetic parameters values of the hydrolase activity were found to be increased significantly on days 45 and 90

($p < 0.0001$) by 3.3- and 2.9-fold for K_M and by 4.3- and 4.7-fold for V_{max}, respectively over the control values (untreated normal rats on day 8 of the study).

In normal BB rats treated with vanadate, the maximal velocities for the hydrolase activity of the G6Pase system were found to be inhibited on day 8 by 15.2% (V_2 group) and by 19.2% (V_3 group), lasted inhibited on day 45 and they were brought back to near normal on day 90. The K_M values in normal BB rats belonging to the V groups were increased on days 8 and 45 (by about 23% for V_2 and 49% for V_3) and they came back to normal on day 90 of the monitoring study.

Table 1

Changes in kinetic parameters of the microsomal hydrolase activity of the G6Pase system in BB rats during a monitoring study (90 days) after a treatment for 7 days with vanadate (V_2 and V_3 groups) comparatively with control group (C)

Rat group	Plasma Glucose (mM)	Days of experiment	K_M (mM)	V_{max} (μmol/min/mg)
C	4.7 ± 0.82	8	0.96 ± 0.09	0.99 ± 0.10
	6.1 ± 1.18	45	1.01 ± 0.58*	1.43 ± 0.25
	19.3 ± 1.38	45	3.15 ± 0.19	4.29 ± 0.48
	5.8 ± 1.07	90	0.97 ± 0.24*	1.85 ± 0.10
	21.7 ± 3.8	90	2.78 ± 0.40	4.69 ± 0.46
V_2	4.9 ± 0.96	8	1.19 ± 0.10	0.84 ± 0.03
	3.6 ± 0.55	45	1.17 ± 0.13	0.84 ± 0.06
	15.5 ± 2.06	45	4.17 ± 0.70	3.85 ± 0.20
	4.8 ± 0.65	90	1.00 ± 0.18	0.92 ± 0.06*
	16.2 ± 2.4	90	3.03 ± 0.54	4.67 ± 0.37
V_3	3.2 ± 0.58	8	1.43 ± 0.25	0.80 ± 0.04
	3.7 ± 0.55	45	1.37 ± 0.20	0.77 ± 0.11
	13.2 ± 2.06	45	5.23 ± 0.75	3.35 ± 0.35
	4.5 ± 0.98	90	1.04 ± 0.14	0.97 ± 0.29*
	14.7 ± 1.9	90	3.46 ± 0.39	4.62 ± 0.32

* no statistically significant differences compared with control rats (on day 8).

The treatment with vanadate provoked a significant decrease (3.35 ± 0.30 *vs.* 4.29 ± 0.48, p 0.02) in the V_{max} values for the hydrolase activity in the diabetic BB rats only for V_3 dose, on day 45, compared with untreated diabetic animals. For the other experimental variants, no statistically significant differences were found for diabetic rats, untreated or treated with vanadate. The values K_M for hydrolase activity in diabetic BB rats treated with vanadate are maintained increased compared with untreated diabetic animals only on day 45 (by 32.4% for V_2 and 66% for V_3).

The kinetic studies on the transferase activity of the G6Pase system were performed with various carbamyl phosphate concentrations at a constant glucose level, as well as with various glucose concentrations at a constant carbamyl

phosphate content, for the microsomes isolated from the rat livers (C, V_1 and V_2 groups) (Table 2). In this study (Fig. 2 and Fig. 3), the K_M values were 4.48 mM for carbamyl phosphate (at 100 mM glucose) and 125 mM for glucose (at 10 mM carbamyl phosphate), while the V_{max} values were found to be 161.3 – 175.4 nmol/ mg/min in normal BB rats at 50 days of age.

Table 2

Effects of vanadate treatment on kinetic parameters of transferase activity of G6Pase system in control and diabetic rats

Rat group	Plasma glucose (mM)	Days of experiment	K_M CP (mM)	K_M Glucose (mM)	V_{max},CP (nmol/min/mg)
C	4.7 ± 0.82	8	4.48 ± 0.43	125.0 ± 15.1	166.4 ± 15.6
	6.1 ± 1.18	45	4.73 ± 0.65*	121.3 ± 14.6	152.8 ± 9.8
	19.3 ± 1.38	45	8.73 ± 0.80	119.2 ± 18.9*	481.2 ± 38.3
	5.8 ± 1.07	90	5.08 ± 0.70*	123.5 ± 13.5*	128.7 ± 7.9
	21.7 ± 3.8	90	9.71 ± 0.40	113.9 ± 14.2*	416.2 ± 25.7
V_1	4.9 ± 0.96	8	5.00 ± 0.42	121.3 ± 12.3*	145.7 ± 5.4
	3.6 ± 0.55	45	4.24 ± 0.33	120.0 ± 16.7*	169.4 ± 15.6*
	15.5 ± 2.06	45	7.82 ± 0.45**	125.6 ± 16.6*	433.3 ± 42.0
	4.8 ± 0.65	90	4.18 ± 0.62	118.3 ± 21.6*	194.7 ± 11.1
	16.2 ± 2.4	90	9.32 ± 0.86**	129.7 ± 12.8*	452.7 ± 28.6
V_2	3.2 ± 0.58	8	6.53 ± 0.60	137.7 ± 9.2*	97.8 ± 10.0
	3.7 ± 0.55	45	4.42 ± 0.58	134.3 ± 11.6*	162.3 ± 17.0*
	13.2 ± 2.06	45	9.05 ± 0.87**	118.5 ± 22.7*	507.3 ± 11.7
	4.5 ± 0.98	90	3.85 ± 0.61	125.0 ± 14.8*	349.0 ± 48.5
	14.7 ± 1.9	90	8.87 ± 1.33**	120.3 ± 18.0*	519.7 ± 23.2

No statistically significant differences compared *with control day (on day 8), **with untreated diabetic rats.

During the monitoring study, in normal BB rats, the V_{max} values for transferase activity decreased with the animal ageing, more significantly on day 90 (p 0.01), while the K_M values for carbamyl phosphate and for glucose were not statistically different. In the kinetic study on the transferase activity, in diabetic untreated group, the maximal velocities and the K_M values for carbamyl phosphate were increased, while the K_M values for glucose were not modified.

The oral administration of vanadate to normal BB rats, for 7 days, leads to a inhibition of transferase activity, proportionally with the dose applied: 12.4% for V_1, 41.2% for V_2 and 100% for V_3 dose. In normal treated BB rats, the V_{max} values of the transferase activity were brought back to near normal on day 45 and increased on day 90, particularly for V_2 dose (p 0.001). The K_M values for carbamyl phosphate increased by 11.6% for V_1 and by 45.8% for V_2 dose, on day 8, are normalized on days 45 and 90, while the K_M values for glucose were not modified.

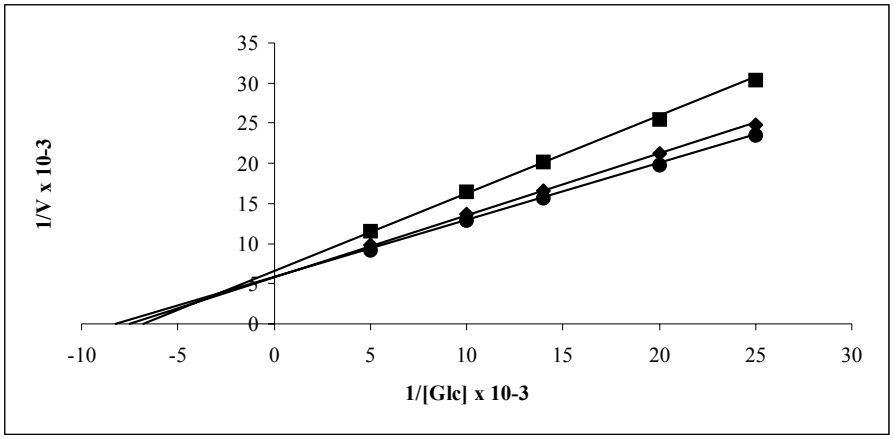

Fig. 2. – The Lineweaver-Burk plots for microsomal glucose-6-phosphotransferase from BB rats belonging to groups C (●), V_1 (♦) and V_2 (■) (for glucose, at 10 mM carbamyl phosphate).

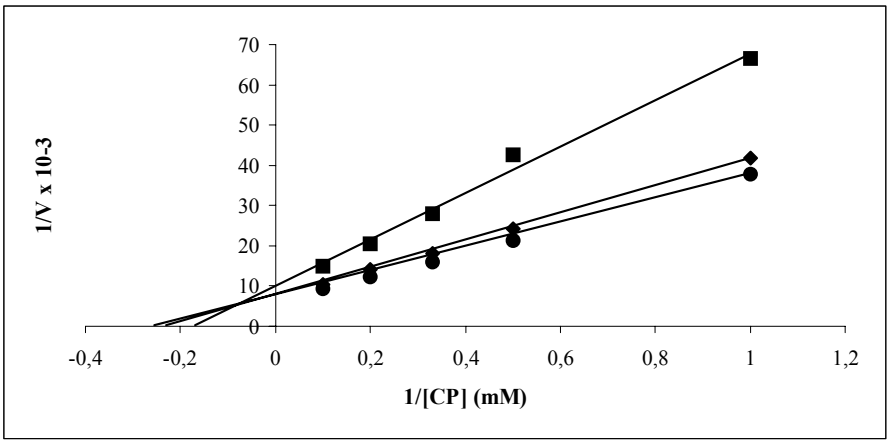

Fig. 3. – The Lineweaver-Burk plots for microsomal glucose-6-phosphotransferase from BB rats belonging to groups C (●), V_1 (♦) and V_2 (■) (for carbamyl phosphate, at 100 mM glucose).

In treated diabetic rats, the values of the kinetic parameters for the transferase activity of the G6Pase system, on days 45 and 90, were not statistically different compared with those found in untreated diabetic animals.

DISCUSSION

This experimental protocol was performed to evaluate the presumed preventive effect of sodium metavanadate on the diabetogenesis process in BB rat.

Our results suggested that vanadate does not prevent diabetes in BB-DP rats, but it can decrease the diabetes incidence and delay the onset. In diabetic rats, the blood glucose levels at the onset diminished with the increase of the vanadate dose (data not shown). To explain these results we studied the hydrolase and phosphotransferase activities of G6Pase system from liver microsomes separated from BB-DP rats treated with three doses of vanadate.

In normal BB rats of prediabetogenic age (about 50 days), the ratio of carbamyl phosphate transferase/ hydrolase V_{max} values, calculated as 0.17, was much less than the ratio found by other researchers, but on different rat strains [15, 20]. At about 130 days of age, in normal BB rats, the hydrolase activity increased by 86.9% and the transferase activity decreased by 22.7%, and this ratio became 0.07. It is possible that this low transferase activity of the liver G6Pase system to be a metabolic trait of these BB rats that develop spontaneous diabetes. These K_M values for the G6Pase system are far removed from the probable hepatic cellular concentrations suggesting that the both enzymatic activities of the G6Pase system are highly responsive to variations in substrate concentration in the physiological range.

In diabetic rats, the both activities of the G6Pase system rose considerably, and the ratio of carbamyl phosphate transferase/hydrolase V_{max} values decreased to 0.112 (on day 45) and 0.088 (on day 90). The K_M values for glucose-6-phosphate and carbamyl phosphate increased, to a more extent for the first substrate, while the K_M values for glucose were not significantly modified, as a result of the increased level of glucose. These responses lead to an enhanced rate of release of glucose from the liver with an adaptation to retain glucose-6-phosphate for hepatic use because of the increased K_M for this substrate.

Our data can by explained by the hypothesis proposed by Nordlie *et al*. [20, 21] that the both activities of the G6Pase system have important roles in the control of blood glucose concentrations and that in diabetes, the control of glucose release at the hepatic level is still maintained by the transferase activity of this system when the insulin-dependent glucokinase level drops precipitously.

After 7 days of vanadate treatment, we found a diminution of the both activities of the G6Pase system in normal BB rats, in the higher extent for the transferase activity. No significant decrease of the hydrolase activity was observed with 0.1 mg/ml vanadate, while at the highest dose (0.7 mg/ml) the inhibition of transferase activity was 100%. The K_M values for glucose-6-phosphate and carbamyl phosphate were found to be increased. Our data are in accord with the results of Singh *et al*. [26] who studied the kinetics of inhibition by vanadate of the G6Pase system from microsomal preparations and permeable isolated hepatocytes.

After cessation of the treatment with vanadate, in normal BB rats, the values of the kinetic parameters for the hydrolase activity are maintained modified on day 45 and were brought back to near normal on day 90. For the transferase activity, the V_{max} values were normalized on day 45 and they were found increased on day

90. The elevated transferase activity after 83 days from the treatment ceasing may explain the success obtained in postponing the diabetes onset. Taking into account the results of Ramanadham *et al.* [23] that the half-life for elimination of vanadium from the bodies of vanadium-fed rats is about 12 days, it is possible that our data to be due to vanadium actions in relation to insulin signaling cascade [22].

CONCLUSION

After diabetes onset the hydrolase and transferase activities of multifunctional G6Pase were elevated 3.8 and 2.2 fold, respectively.

Treatment with metavanadate decreased the both activities, proportionally with the dose applied, but was a more potent inhibitor of carbamyl phosphate: glucose phosphotransferase activity.

Elevated level of carbamyl phosphate: glucose phosphotransferase activity after 83 days from the treatment ceasing may explain the success obtained in postponing of diabetes onset.

Vanadate treatment restored glucose-6-phosphate level in the liver and decreases hyperglycemia.

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