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Insulin-like Effects of Vanadate on Glucokinase Activity and Fructose 2,6-Bisphosphate Levels in the Liver of Diabetic Rats*

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Streptozotocin diabetic rats showed more than a 4-fold increase in blood glucose levels, whereas hepatic glycogen, fructose 2,6-bisphosphate concentration, and 6-phosphofructo-2-kinase activity were decreased. The "total" 6-phosphofructo-2-kinase and the "active" (nonphosphorylated) form of the enzyme were decreased to a different extent, resulting in a fall of the "active"/"total" activity ratio. Vanadate administration for a 2-week period restored the altered values in the diabetic rats without modifying significantly in the control animals any of the parameters studied. Glucokinase activity was essentially lacking in the diabetic animals, and vanadate treatment restored the activity to about 65% of its control value, a good correlation between the recovery of the enzyme and the blood glucose level being observed. These results show an insulin-like effect of vanadate in the whole animal and suggest that insulin and vanadate possess similar actions on hepatic intracellular events.

Although the effects of vanadium have received increased attention (for review see Refs. 1 and 2), its function still remains unclear. Biochemically, vanadium compounds generally affect systems that utilize ATP and enzymes that catalyze phosphotransferase and phosphohydrolase reactions (1–8).

In intact cells, vanadium compounds possess insulin-like effects. They mimic both the glucose transport-dependent and the intracellular actions of insulin in isolated rat adipocytes (9–14) and in skeletal muscle (15). They enhance glycogen synthesis in rat hepatocytes (16) and inhibit ACTH[1]-induced lipolysis in rat adipocytes (11). Furthermore, vanadate elicits a mitogenic response in human fibroblasts and in the 3T3 and 3T6 cell lines (17, 18), and it interacts synergistically with growth factors such as epidermal growth factor, insulin, and insulin-like growth factor II (17–19).

In the whole animal, Heyliger *et al.* (20) have shown that oral administration of vanadate to diabetic rats normalized the high blood glucose concentration and prevented the decline in cardiac performance due to diabetes, suggesting that vanadate has an insulin-like effect on glucose metabolism "*in vivo.*"

In diabetes there are profound metabolic changes in liver carbohydrate metabolism, which likely flow primarily from altered hormonal regulation of metabolic signals such as fructose-2,6-P_2. This bisphosphorylated metabolite, by acting as allosteric effector of both phosphofructokinase and fructose-1,6-bisphosphatase, regulates the flux over the glycolytic/gluconeogenic pathways, and thereby it can also control hepatic lipid metabolism and ketogenesis (21–24). The concentration of fructose-2,6-P_2 in liver is determined by nutritional and hormonally induced changes in the activity of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase responsible for its synthesis and degradation. The regulation of the enzyme is a complex function of both the influence of substrates and effectors and its covalent modification via phosphorylation/dephosphorylation (21–24). Phosphorylation by the cyclic AMP-dependent protein kinase results in inhibition of the kinase and activation of the bisphosphatase, while dephosphorylation results in opposite changes in the two activities (25). In addition, the total enzymatic activity is dependent on the rates of synthesis and degradation of the enzyme (26).

It has been found that in the liver of alloxan diabetic rats the levels of fructose-2,6-P_2 and 6-phosphofructo-2-kinase activity are decreased (27), and the degree of phosphorylation of the bifunctional enzyme is increased, a good correlation between fall of hepatic fructose-2,6-P_2, ketonemia, and glycemia being observed (28). Insulin administration normalizes all the values (27, 28).

The present work was undertaken to study the effects produced by vanadate administration on glycemia and on the levels of glycogen, fructose-2,6-P_2 and 6-phosphofructo-2-kinase activity in the liver of diabetic rats. It also reports the effect of vanadate administration on the induction of the hepatic glucokinase activity, which is almost absent in diabetes (29).

EXPERIMENTAL PROCEDURES

Chemicals—Sodium orthovanadate (Na_3VO_4) was obtained from Fisher. Enzymes and biochemical reagents were from either Boehringer Mannheim or Sigma. All other chemicals were of analytical grade.

Treatment of Animals—The general features of the experiment were as described by Heyliger *et al.* (20). Male Sprague-Dawley rats weighing 190–210 g were made diabetic by intravenous injection of streptozotocin (60 mg/kg) dissolved in 0.5 ml of 50 mM sodium citrate, 0.15 M NaCl, pH 4.5. Controls received buffer injections only. One week after diabetes induction blood samples were collected from the tail vein and were determined for concentration of glucose. The animals were judged diabetics if blood glucose levels exceeded 350 mg/dl and were randomly divided into four groups: control; vanadate-treated controls; streptozotocin-injected; and vanadate-treated streptozotocin-injected. The controls and the streptozotocin-injected group drank a 0.5 g/100 ml NaCl solution. The vanadate-treated animals group drank a 0.5 g/100 ml NaCl solution containing sodium orthovanadate (0.7 mg/ml) prepared freshly everyday. The vanadate intake was 77 ± 10 mg/kg/day for the controls and 61 ± 8 mg/kg/day for

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[1] The abbreviations used are: ACTH, corticotropin; fructose-2,6-P_2, fructose 2,6-bisphosphate; Mes, 4-morpholineethanesulfonic acid.

TABLE I

Body weight, food and fluid intake, and liver vanadium content of experimental rats

General features of the experiment are described under "Experimental Procedures." Results are expressed as means ± S.E. for the number of animals in parentheses, and the significance of differences was tested by Student's *t* test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *versus* untreated controls.

Group	Body weight	Food intake	Fluid intake	Liver vanadium
	g	*g/day*	*ml/day*	*μg/g*
Control				
Untreated (5)	320 ± 4	24 ± 0.6	41 ± 5	<0.05
Vanadate-treated (8)	296 ± 10*	20 ± 2*	23 ± 3*	0.94 ± 0.10***
Streptozotocin-injected				
Untreated (8)	261 ± 12***	45 ± 2***	268 ± 18***	<0.05
Vanadate-treated (11)	269 ± 12**	19 ± 2*	25 ± 3*	1.16 ± 0.28***

TABLE II

Effects of diabetes and vanadate administration on blood glucose and on the hepatic levels of glycogen and fructose-2,6-P_2

General features of the experiment are described under "Experimental Procedures." Results are expressed as means ± S.E. for the number of animals in parentheses, and the significance of differences was tested by Student's *t* test: *** $p < 0.001$ *versus* untreated controls.

Group	Blood glucose	Glycogen	Fructose-2,6-P_2
	mg/dl	*mg/g*	*nmol/g*
Control			
Untreated (5)	116 ± 7	41.8 ± 4.3	8.6 ± 1.2
Vanadate-treated (8)	104 ± 6	44.4 ± 3.4	6.7 ± 1.1
Streptozotocin-injected			
Untreated (8)	486 ± 25***	17.8 ± 2.2***	0.5 ± 0.1***
Vanadate-treated (11)	125 ± 5	45.7 ± 2.6	11.9 ± 1.5

the diabetic rats. Diabetes was assessed periodically by a test for glucosuria (Urotron^R from Boehringer Mannheim). The animals were killed by decapitation when the glucosuria levels of the vanadate-treated diabetic group were normalized (2 weeks after the beginning of vanadate treatment). Blood samples were collected at the time of the death, and the livers were removed and fastly freeze-clamped in liquid N_2.

Assays of Metabolites and Enzymes—Fructose-2,6-P_2 was extracted and measured as described in Ref. 30. The "total" 6-phosphofructo-2-kinase activity and the "active" form of the enzyme corresponding to the activity of the nonphosphorylated form of the enzyme were measured as described in Ref. 31 on the basis of their different kinetic properties (32). Liver samples were homogenized in 20 volumes of ice-cold 20 mM P_i, 10 mM EDTA, 100 mM KF, 1 mM dithiothreitol, pH 7.1, and centrifuged at 27,000 × *g* for 15 min at 4 °C. Portions of the resulting supernatants were incubated for 10 min at 30 °C in the presence of the constituents described below in a final volume of 0.25 ml. 6-Phosphofructo-2-kinase was assayed in the presence of 5 mM ATP, 7 mM $MgCl_2$, and 100 mM KCl. Furthermore, 50 mM Tris, 1 mM P_i, 5 mM fructose-6-P, and 17.5 mM glucose-6-P were also present, and the pH was adjusted to 8.5 for determination of the total activity; 50 mM Mes, 5 mM P_i, 1 mM fructose-6-P, and 3.5 mM glucose-6-P were present and the pH was adjusted to 6.6 for the determination of the active form of enzyme. Glucokinase activity was calculated as the difference between the glucose phosphorylation capacity at 100 and 0.5 mM glucose, measured using the continuous assay described by Davidson and Arion (33). One unit of activity is defined as the amount of enzyme that transforms 1 μmol of substrate/min. Blood glucose was asssayed as described in Ref. 34. Liver glycogen was isolated as described in Ref. 35, and the amount of glucose produced by acid hydrolysis was determined as in Ref. 34. Vanadium was extracted and measured as described in Ref. 36.

RESULTS

The four groups of animals used in this study showed different fluid and food intake, as summarized in Table I. Body weights of the diabetic rats were significantly lower than body weights of the controls. The fluid and the food intake of the diabetic vanadate-untreated rats were larger than those of the control rats. Animals that drank the vanadate solution consumed significantly less fluid and food than control animals. Similar results were reported by Heyliger *et al.* (20). The content of vanadium in the liver of untreated animals was less than 0.05 μg/g, while the vanadate-treated animals showed values of 1 μg/g (Table I).

The animals made diabetic with streptozotocin showed more than a 4-fold increase in blood glucose levels and a 60% decrease in hepatic glycogen concentration (Table II). In contrast, glycemia and hepatic glycogen concentration of the diabetic vanadate-treated rats were not significantly different from those of the controls. Liver fructose-2,6-P_2 concentration decreased to about 6% of the control values in the diabetic untreated animals, and the treatment with vanadate restored the control levels of this metabolite. Administration of vanadate to control rats did not modify significantly either the blood glucose concentration or the hepatic glycogen and fructose-2,6-P_2 levels.

Table III summarizes the changes induced by diabetes and by vanadate treatment in the hepatic glucokinase activity and in the total and active (nonphosphorylated) 6-phosphofructo-2-kinase activities. It also summarizes the changes in the active/total 6-phosphofructo-2-kinase activity ratio, which reflects the degree of phosphorylation of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (31, 32). As shown, both the total and the active 6-phosphofructo-2-kinase activities were significantly decreased in the diabetic animals. However, the decrease produced in the active form of the enzyme was higher than that produced in the total 6-phosphofructo-2-kinase activity, resulting in a fall in the active/total activity ratio. Vanadate treatment of the diabetic rats restored both the total and the active 6-phosphofructo-2-kinase activities. In the diabetic animals glucokinase activity was essentially lacking, and vanadate treatment restored the activity to about 65% of the control value. Treatment of the control rats with vanadate did not modify any of the activities measured.

As shown in Fig. 1, there is a good correlation between the hepatic glucokinase activity and the severity of the diabetes, estimated by the blood glucose level. In order to obtain partially recovered values of glucokinase, four diabetic vanadate-treated animals were killed when glucosuria was still present. These animals had normalized their hepatic glycogen and fructose-2,6-P_2 levels.

DISCUSSION

The results herein reported clearly show that vanadate mimics insulin effects *in vivo*, reducing the high blood glucose and restoring glycogen and fructose-2,6-P_2 levels, and glucokinase and 6-phosphofructo-2-kinase activities in the liver of diabetic animals. The mechanism by which vanadate restores glycemia is probably related to the hepatic effects of the compound, in addition to those reported in peripheral tissues (9–16). Previous results (20) exclude an indirect mechanism

TABLE III

Effects of diabetes and vanadate administration on hepatic glucokinase and 6-phosphofructo-2-kinase activities

General features of the experiment are described under "Experimental Procedures." Results are expressed as means ± S.E. for the number of animals in parentheses, and the significance of differences was tested by Student's *t* test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *versus* untreated controls.

Group	Glucokinase	6-Phosphofructo-2-kinase		
		Active	Total	Activity ratio
	units/g	*milliunits/g*		
Control				
Untreated	3.18 ± 0.28 (5)	4.0 ± 0.40 (5)	6.2 ± 0.63 (5)	0.64 ± 0.08 (5)
Vanadate-treated	2.74 ± 0.15 (8)	4.3 ± 0.51 (8)	6.7 ± 0.46 (8)	0.64 ± 0.05 (8)
Streptozotocin-injected				
Untreated	0.05 ± 0.01*** (8)	0.9 ± 0.17*** (4)	3.3 ± 0.70* (4)	0.29 ± 0.01** (4)
Vanadate-treated	2.08 ± 0.16*** (10)	4.9 ± 0.41 (6)	6.5 ± 0.13 (6)	0.75 ± 0.06 (6)

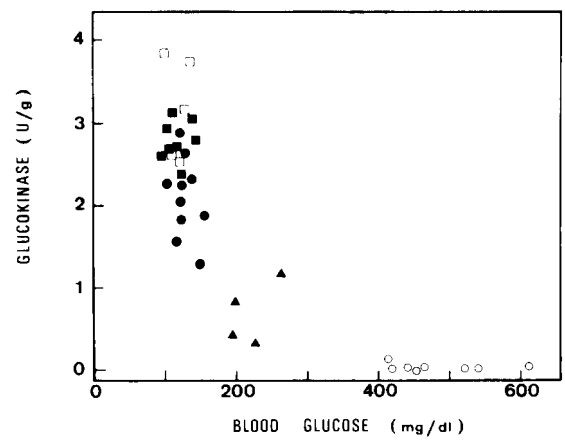

FIG. 1. **Correlation between glycemia and hepatic glucokinase activity.** Each point corresponds to one animal. The groups were described under "Experimental Procedures": □, controls; ■, vanadate-treated controls; ○, streptozotocin-injected; ●, streptozotocin-injected and vanadate-treated. Four streptozotocin-injected vanadate-treated animals were killed when glucosuria levels were between diabetic and normal values (▲).

mediated by an increase in insulin secretion.

It is believed that the modulation of glucokinase activity, which plays a major role in the maintenance of glucose homeostasis (29), arises from changes in the amount of the enzyme present within the cell. Both studies *in vivo* (29, 37) and using primary cultures of rat hepatocytes (38, 39) have shown that the induction of glucokinase by insulin is the result of an increase in the rate of synthesis of the enzyme. In streptozotocin-induced diabetic rats the low levels of glucokinase synthesis and activity correlated with a low level of mRNA coding for glucokinase (40). Administration of insulin increased translatable glucokinase mRNA and enhanced the rate of synthesis and the activity of the enzyme (29, 37, 40). Our results show that, similar to insulin, vanadate treatment restores the decreased glucokinase activity in the liver of diabetic rats. Vanadate could develop this insulin-like effect by inducing the synthesis of the enzyme. This is also probably true for the increase in the total 6-phosphofructo-2-kinase activity of vanadate-treated diabetic rats.

The restoration of the active/total 6-phosphofructo-2-kinase activity ratio produced by vanadate could be explained by a decrease in the degree of phosphorylation of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Such a decrease would result in activation of its kinase and inhibition of its bisphosphatase activities, and as a consequence fructose-2,6-P_2 concentration would increase (21–25). Similar effects have been obtained by insulin treatment of diabetic rats (27, 28, 41). The increase in the hepatic concentration of fructose-2,6-P_2, by acting on the enzymes involved in the fructose-6-P/fructose-1,6-P_2 cycle (21–24), would shift the predominating gluconeogenic flux of diabetes to the glycolytic flux of the vanadate-treated animals.

The effect of vanadate on fructose-2,6-P_2 concentration cannot be explained by a direct action on 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase activity, since it has been recently reported that "*in vitro*" vanadate inhibits the kinase activity of the purified bifunctional enzyme without modifying its phosphatase activity (8). The discrepancies between the *in vivo* and *in vitro* experiments could be explained by the distinct effects of the different forms of vanadium (vanadate and vanadyl). It is known that vanadate enters cells by the anion transport mechanism and it is reduced to vanadyl ions (1, 2). It has been suggested that the form of vanadium with insulin-like effects observed in whole cell experiments is the vanadyl ion, whereas vanadate acts as an inhibitor of the formation of phosphoprotein intermediates (9–11, 42).

Diabetic animals possess low hepatic glycogen content, and the activation of glycogen synthase and accumulation of glycogen in response to glucose are impaired in livers and hepatocytes of diabetic rats. The exact nature of the defect has not been established, although the decrease in synthase phosphatase activity emerges as a decisive event (43, 44). In agreement with the stimulation of glycogen synthesis by vanadate in isolated rat hepatocytes found by others (16), the results now reported show that vanadate treatment of diabetic rats restores the hepatic levels of glycogen, possibly through activation of glycogen synthase as demonstrated in other cells (12, 13). A recent report of Bosch *et al.* (45) describes inactivation of glycogen synthase and activation of glycogen phosphorylase by vanadate in isolated rat hepatocytes, concluding that in liver vanadate does not have insulin-like activity. The discrepancies between these results (45) and both those from Tolman *et al.* (16) and the results herein reported could be explained through the following facts: (*a*) the different effects of vanadyl and vanadate, as described above; (*b*) the influence of the period of treatment in the different experimental approaches; (*c*) the accumulation of vanadate inside the cells, in addition to vanadyl, when high concentrations of vanadate are used (42). In our experimental conditions the concentration of vanadium in the liver of treated animals attains values of 1 μg/g (Table I), corresponding to about 20 nmol/g vanadium.

The cellular mechanisms of vanadate effects are not fully understood, although several biochemical actions might explain its insulin-like effects. Vanadate, as well as insulin, increases the autophosphorylation and the tyrosine kinase activity of the insulin receptor (12, 13). However, a recent

report proposes that vanadate stimulates glucose transport in adipocytes acting at a level distal to the insulin receptor (14). It could also act by increasing phosphorylation of other key proteins by either activation of the tyrosine kinase (46) or inhibition of the phosphotyrosine phosphatase (47) activities. The spontaneous esterification of tyrosine residues with vanadate could be an alternative mechanism of vanadate actions (48). In addition, vanadium compounds could act by mediating changes in the concentration of cyclic AMP (1, 2) and Ca^{2+} ions (49).

In conclusion, the results herein and elsewhere reported (20) show an insulin-like effect of vanadate in the whole animal and suggest that insulin and vanadate possess similar actions on hepatic intracellular events. However, further studies are required to elucidate the mechanism of the cellular actions of vanadate. This information could provide valuable insight into the mechanism of the insulin action.

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