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IMPAIRED ANTIOXIDANT STATUS IN DIABETIC RAT LIVER

EFFECT OF VANADATE

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Abstract—*In vivo* effects of vanadate on the antioxidant status of control and alloxan diabetic rats liver were examined. The increased oxidative stress during diabetes caused a decline in the activities of glutathione peroxidase (GPx), catalase (CAT), CuZn superoxide dismutase (CuZn-SOD) and Mn-superoxide dismutase (Mn-SOD) in the liver. Reduced glutathione (GSH) was also depleted, but the level of oxidized glutathione and glutathione reductase activity remained unchanged in the livers of diabetic rats. Vanadate treatment of diabetic rats (0.6 mg/mL in drinking water) resulted in almost complete restoration of GPx and Mn-SOD but caused only a partial restoration of CuZn-SOD. However, CAT and GSH were found to be lowered further in vanadate-treated diabetic rats as compared to untreated diabetic rat. Similar decreases in CAT and GSH levels were also observed in the vanadate-treated controls. These results suggest that vanadate, an insulin-mimetic agent, effectively normalized hyperglycemia, but unlike insulin, could not completely restore the altered endogenous defence mechanisms in diabetic liver.

The biological role of vanadate has not been firmly established [1, 2]. Recent studies, have shown that vanadate is a potent insulin-mimetic agent and promotes glucose uptake [3, 4], glucose oxidation [5] and activation of glycogen synthase in rat liver [6] and adipocytes [7]. Oral administration of vanadate to alloxan and streptozotocin diabetic rats restores elevated blood glucose level and several carbohydrate-metabolizing enzymes to control values [6, 8, 9]. Recently, we have shown that vanadate treatment normalizes glycolytic enzymes and malic enzyme in both insulin-dependent and -independent tissues [9] and prevents renal hypertrophy in diabetic rats [10]. However, the mechanism for its insulin-like actions is still not clear. Evidence is accumulating which suggests that toxic reactive oxygen-derived free radicals (superoxide, peroxide and hydroxyl radicals) play a crucial role in diabetes [11–14]. During diabetes, persistent hyperglycemia causes increased production of free radicals via autoxidation of glucose [12] and also via non-enzymatic protein glycation [13] that may lead to disruption of cellular functions and oxidative damage to membranes [11]. The levels of these reactive oxygen species are controlled by antioxidant enzymes namely, superoxide dismutase (SOD†) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6), glutathione peroxidase (GPx) (EC 1.11.1.9) and nonenzymatic scavengers like reduced glutathione (GSH). Marked alterations in antioxidant enzyme activities and tissue GSH concentrations have been reported in diabetes [11, 14]. Thus, the tissue antioxidant status seems to emerge as an important factor in the etiology of diabetic complications.

Wohaieb and Godin [14] demonstrated previously that insulin treatment of diabetic rats resulted in almost complete reversal of all the foregoing alterations in tissue antioxidant status. Hence, an antioxidant role was presumed for vanadate, in view of its insulin-mimetic properties of correcting various carbohydrate-metabolizing enzymes. The present study was, therefore, specifically aimed to examine whether, like insulin, vanadate treatment can normalize the disturbed antioxidant status in experimental diabetes.

MATERIALS AND METHODS

Materials. Sodium orthovanadate, alloxan monohydrate, enzymes, coenzymes and substrates were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All the other chemicals were standard analytical grade laboratory reagents.

Treatment of animals. Male Wistar rats (200–220 g; age 10–12 weeks) were starved for 24 hr and diabetes was induced by a single subcutaneous injection of alloxan monohydrate (200 mg/kg body weight) dissolved in 0.15 M sodium acetate buffer, pH 4.5. Another group of rats received a corresponding volume of sodium acetate buffer and served as controls. The alloxan-induced diabetic rats were injected i.p. with 2 IU of protamine-zinc insulin for the next 7 days. Insulin was then withdrawn and both groups of rats were randomly divided into four groups: control, vanadate-treated control, diabetic

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† Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Table 1. General parameters of the four experimental groups

	Control	Control + vanadate	Diabetic	Diabetic + vanadate
Body weight (g)	263 ± 9	250 ± 8	203 ± 6*	211 ± 7*
Liver weight (g)	10.4 ± 0.7	10.2 ± 0.5	7.9 ± 0.4‡	7.8 ± 0.4†
Protein content (mg/g wet weight)	159 ± 5	163 ± 6	161 ± 7	156 ± 5
Fluid intake (mL)	35 ± 4	33 ± 4	190 ± 8*	32 ± 3
Vanadium intake (mg/kg/day)	—	79 ± 10	—	91 ± 9
Blood glucose (mg/100 mL)	106 ± 6	100 ± 7	415 ± 17*	104 ± 7
Plasma insulin (μU/mL)	16.8 ± 1.9	10.9 ± 0.9‡	4.5 ± 0.8*	5.5 ± 0.4*

The treated control and diabetic rats received sodium orthovanadate (0.6 mg/mL)-supplemented water.
Values are means ± SE of six rats.
Significance of difference was assessed by Student's *t*-test. P values are shown as *$P < 0.001$, †$P < 0.01$ and ‡$P < 0.05$ vs control.

and vanadate-treated diabetic rats. Sodium orthovanadate (0.6 mg/mL) was given to the treated rats in drinking water which also contained 80 mM NaCl. As described earlier [8, 9], NaCl was included to reduce the toxicity of vanadate. The untreated control and diabetic rats received drinking water supplemented with 80 mM NaCl. All animals were maintained for 3 weeks and had access to water and food *ad lib*. At the end of the treatment period, blood was collected by eye vein in heparin and rats were killed by cervical dislocation.

Determination of GSH and oxidized glutathione (GSSG). Livers were rapidly excised, blotted dry and immediately homogenized in ice-cold 2 M perchloric acid, 4 mM EDTA using a Potter–Elvehjem homogenizer fitted with a teflon plunger. Homogenates were then centrifuged at 1000 *g* for 10 min. The clear extracts were neutralized with 2 N KOH, and GSH and GSSG were measured enzymatically as described by Griffith [15] on the same day. For GSH estimation, the reaction mixture contained 0.21 mM NADPH, 0.6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 0.5 U glutathione reductase in 125 mM sodium phosphate buffer (pH 7.5), and 6.3 mM EDTA. The rate of 5,5′-dithiobis-(2-nitrobenzoic acid) reduction was continuously followed at 412 nm and GSH content was determined by comparing the rate observed using a standard curve generated with a known amount of GSH. For GSSG, GSH was derivatized by adding 2-vinyl pyridine (2 μL/mL extract) and was kept for 1 hr at 25°. The GSSG content was then measured directly as described for GSH.

Enzyme assays. For enzyme assays, livers were homogenized in 10 mM potassium phosphate buffer pH 7.4, supplemented with 30 mM KCl. Homogenates were then centrifuged at 1000 *g* for 10 min. For SOD measurement, the supernatant was treated with Triton X-100 (1% final concentration) and left for 30 min on ice to solubilize both CuZn-SOD and Mn-SOD. The SOD activity was analysed by the method of Marklund and Marklund [16] based on the ability of the enzyme to inhibit autoxidation of pyrogallol and expressed as units of SOD per gram tissue (one unit is the amount of enzyme that causes half-maximal inhibition of pyrogallol autoxidation). For measurement of Mn-SOD, 10 mM potassium cyanide was used to inhibit CuZn-SOD.

Samples for CAT were prepared by incubating the supernatant with ethanol (10 μL/mL) for 30 min in ice and then Triton X-100 was added to a final concentration of 1%. CAT assay was performed by the method of Aebi [17] and the activity was expressed as IU/g tissue × 10^3. In the 1% Triton X-100-treated samples GPx and glutathione reductase (GR) (EC 1.6.4.2) were measured by the method of Lawrence and Burk [18] and Erden and Bor [19], respectively. One unit of GPx and GR activity was defined as one micromole of NADPH oxidized per minute at 25°. All measurements were carried out using a Beckman DU-68 spectrophotometer.

Blood glucose, plasma insulin and protein determination. Blood glucose was measured by an enzyme coupled assay using glucose-6-phosphate dehydrogenase and hexokinase as described [20]. The plasma insulin levels were quantitated by radioimmunoassay kit supplied by BARC, India. Protein content was estimated in the liver extracts by the method of Bradford [21].

RESULTS

The general parameters as observed in the four experimental groups are shown in Table 1. Diabetes was characterized by hyperglycemia (4-fold increase) and hypoinsulinemia (73% decrease). Vanadate administration (0.6 mg/mL), within 3–4 days, normalized the elevated blood glucose level but was unable to restore the decreased plasma insulin level of diabetic rats. If the vandadate treatment was withdrawn from the diabetic rats, hyperglycemia was found to recur within 72 hr. Vanadate treatment reversed the increased water intake of diabetic rats to normal values with no signs of dehydration. The body weight of treated diabetic rats remained almost equal to that of untreated diabetic rats. Vanadate treatment caused a 35% decrease in plasma insulin level of control rats that could be due to an increased tissue responsiveness for insulin, as suggested earlier [10, 22]. There was no change in the total protein content/g liver after treatment as compared to the control values.

Table 2 depicts the antioxidant status of liver in various experimental groups. Measurement of free radical-scavenging enzymes in diabetic rats showed a generalized decrease in the activities of GPx, CAT,

Table 2. GPx, GR, CAT, CuZn-SOD and Mn-SOD activities and GSH and GSSG levels in the livers of different experimental groups

	Control	Control + vanadate	Diabetic	Diabetic + vanadate
Enzyme activities (U/g)				
GPx (6)	22.1 ± 1.6	20.1 ± 1.8	15.4 ± 1.5§	21.8 ± 1.9
GR (6)	6.8 ± 0.2	6.0 ± 0.2§	6.6 ± 0.2	6.4 ± 0.3
CAT* (6)	34.0 ± 2.2	27.4 ± 2.8	24.0 ± 1.7‡	20.9 ± 2.1‡
CuZn-SOD (8)	2238 ± 276	2077 ± 270	1333 ± 174§	1682 ± 184
Mn-SOD (8)	225 ± 22	213 ± 17	162 ± 18§	220 ± 17
Metabolite levels (μmol/g)				
GSH (6)	5.5 ± 0.4	4.2 ± 0.5§	3.8 ± 0.4§	3.0 ± 0.3†
GSSG (6)	0.18 ± 0.03	0.16 ± 0.02	0.19 ± 0.03	0.17 ± 0.03

The treated control and diabetic rats received vanadate-supplemented water.
Values are means ± SE.
Number of animals (N) is given in parenthesis.
* Activity is expressed in $U/g \times 10^3$.
Significance of difference was assessed by Student's *t*-test. P values are shown as †$P < 0.001$, ‡$P < 0.01$ and §$P < 0.05$ vs control.
Other details are given in Materials and Methods.

CuZn-SOD and Mn-SOD as also reported earlier [14]. Oral administration of vanadate to diabetic rats almost completely normalized the 30% depressed GPx level. In diabetic rats, a 28% decline in the Mn-SOD was observed that returned to normal value following vanadate administration. However, the treatment only partially restored the 40% decreased diabetic state CuZn-SOD level.

In the diabetic rat liver, a 29% and 31% decrease in CAT and GSH levels was observed, respectively. Vanadate treatment of diabetic rats was, however, found to be totally ineffective in the restoration of the CAT and GSH level. On the other hand, the depressed CAT and GSH levels in the diabetic rats were lowered further in the vanadate-treated diabetic rats. A similar pattern was observed in the treated control rats where CAT and GSH levels were decreased from the control values (19% and 24%, respectively). No effect of vanadate was seen on the GR activity which remained unaltered during diabetes. GSSG levels were also found to be unchanged in the diabetic and treated rats.

DISCUSSION

Vanadium is now considered an essential nutritional trace element and has therapeutic value in pharmacological doses, but is toxic in excess and causes stimulation of H_2O_2 production and lipid peroxidation [2]. Our studies, along with those of others, have demonstrated that vanadate mimics many of the actions of insulin and its oral administration improves the altered glucose homeostasis and other metabolic disorders of the diabetic state [4–10].

There is now ample evidence to suggest that, in certain pathological states like diabetes, the increased production and ineffective scavenging of toxic reactive oxygen species may play a crucial role in determining tissue injury [11–14]. Diabetes causes a depression in overall liver antioxidant status making it more vulnerable to oxygen radical attack which may cause oxidative damage to membranes and alterations in subcellular organelle structural and functional integrity [14]. The decreased levels of GPx, CAT, CuZn-SOD and Mn-SOD in the diabetic state may be due to inactivation caused by reactive oxygen species [23]. This decrease in antioxidant enzymes may also be due to the emaciation observed in the diabetic rats [14]. In diabetes, non-enzymatic glycation due to persistent hyperglycemia may also inactivate the antioxidant enzymes as shown previously with SOD by Arai *et al.* [24]. Insulin treatment of diabetic rats was shown previously to normalize all the foregoing alterations in the antioxidant status [14].

In view of the various insulin-mimetic effects of vanadate, an attempt was made to study the efficacy of vanadate treatment in controlling the altered antioxidant status of the liver of diabetic rats. The dose (0.6 mg/mL) of sodium orthovanadate (Na_3VO_4) was carefully chosen since it had been established previously as optimal for normalizing hyperglycemia and for exerting insulin-mimetic effects in diabetic rats [8–10].

GPx has a key role in enzymatic defence systems and acts on peroxides (H_2O_2, lipid or organic peroxides) to remove them. In the present investigation, it was observed that vanadate therapy could effectively normalize the GPx and Mn-SOD level but it only partially restored the level of CuZn-SOD. This suggests that the increased oxidative stress during diabetes (involving increased O_2^- and H_2O_2 production) is controlled to some extent only. Paradoxically, we found that, instead of restoration, the decreased levels of CAT and GSH in diabetic rats had declined further in vanadate-treated diabetic rats. This observation was not expected from an insulin-mimetic agent like vanadate. A similar pattern of decrease in CAT and GSH was seen in vanadate-treated controls. However, the present results are contradictory to those of Sekar *et al.* [25] who observed the restoration of both CAT and GSH in vanadate-treated diabetic rats, which could be due to the low dose of orthovanadate (0.3 mg/mL) used in their study. Degani *et al.* [26] showed by electron paramagnetic resonance studies that externally applied vanadate (V V, VO_4) is reduced

intracellularly to vanadyl ($V^{IV}O_2$) and binds endogenously with GSH to form vanadyl–GSH complexes which may produce its insulin-like effects. Thus, the decrease observed in the level of GSH in treated control and diabetic rats may be due to its consumption in the formation of such vanadyl–GSH complexes. The downward trend in CAT level seen in treated control and diabetic rats as compared to their counterparts cannot be explained at this stage. Vanadate may produce some other form of free radical or factor leading to site-specific inactivation of CAT. It is also possible that vanadate administration in the dose applied has some other role in the biological system independent of its insulin-mimetic effects. This is likely as vanadium is a transition element with variable oxidation states (+3, +4, +5, +7) and theoretically should influence the tissue redox state and, in turn, the antioxidant enzymes.

In conclusion, the present investigation indicates that vanadate therapy is only partially effective in controlling the impaired antioxidative system of diabetic rats and is accompanied by some adverse effects as, unlike insulin, it fails to normalize CAT and GSH levels which are of crucial importance for the host defence. On the basis of the foregoing study it is suggested that vanadate treatment accompanied by some known antioxidant (e.g. flavanoids [27]), to improve the concomitant defence system disturbances, may produce better therapeutic results. More incisive studies are warranted to explore other insulin-like effects of vanadate that may also elucidate the mechanism of vanadate action at the molecular and cellular level.

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