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Oral Tungstate Treatment Improves Only Transiently Alteration of Glucose Metabolism in a New Rat Model of Type 2 Diabetes

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It has been shown that tungstate is an effective hypoglycemic agent in several animal models of diabetes. In this study, we examined the effectiveness of oral tungstate treatment in a new experimental diabetic syndrome, induced by streptozotocin (STZ) and nicotinamide in adult rats, that shares several features with human type 2 diabetes. Sodium tungstate was administered in the drinking water (2 mg/mL) of control and diabetic rats for 15, 30, 60, and 90 d. Glucose metabolism was explored in vivo by intravenous glucose tolerance test. Insulin secretion and action were assessed in vitro in the isolated perfused pancreas and isolated adipocytes, respectively. Two weeks of tungstate treatment did not modify the moderate hyperglycemia of diabetic rats but reduced their intolerance to glucose, owing to an enhancement of postloading insulin secretion. However, this effect was transient, since it declined after 30 d and vanished after 60 and 90 d of tungstate administration, whereas a trend toward a reduction in basal hyperglycemia was observed on prolonged treatment. Oral tungstate was unable to modify glucose-stimulated insulin secretion in the isolated perfused pancreas, as well as muscle glycogen levels, hepatic glucose metabolism, and insulin-stimulated lipogenesis in isolated adipocytes. Nevertheless, the decreased insulin content of pancreatic islets of diabetic rats was partially restored on prolonged tungstate treatment. In conclusion, in the STZ-nicotinamide model of diabetes, tungstate was unable to permanently correct the alterations in glucose metabolism, despite some indirect evidence of a trophic effect on β-cells. The ineffectiveness of tungstate could be related to the absence, in this diabetic syndrome, of relevant metabolic alterations in the liver, which thus appear to constitute the major target of tungstate action.

Key Words: Experimental diabetes; tungstate; glucose tolerance test; insulin secretion; insulin resistance; isolated adipocytes.

Introduction

It has been reported that a 2-wk oral tungstate treatment reduced the severe hyperglycemia of streptozotocin (STZ)-diabetic rats *(1)*, and normalized as well the moderate hyperglycemia of neonatally injected STZ (nSTZ) rats, a model of type 2 diabetes *(2)*. Furthermore, glucose metabolism improved in Zucker diabetic fatty rats *(3)* and in STZ-diabetic rats *(4)* on a prolonged tungstate treatment (for 2 and 8 mo, respectively). In all these cases, the effect of tungstate appeared mainly mediated by a restoration of hepatic glucose metabolism, more or less markedly impaired in such diabetic animals *(1–4)*. On the basis of these results, it was suggested that tungstate could be helpful in the treatment of both type 1 and 2 diabetes.

In the present study, we determined the effects of oral tungstate treatment in a novel experimental model of type 2 diabetes, obtained in adult rats by the combined administration of STZ and a partial protective dose of nicotinamide (NA). This model is characterized by a 40% reduction in β-cell mass *(5)*, which results in moderate and stable hyperglycemia, glucose intolerance, altered but still present ability of β-cells to respond to glucose, and preserved responsiveness to tolbutamide *(6)*, thus sharing a number of similarities with human type 2 diabetes. Actually, the insulin responsiveness to glucose and sulfonylureas, which is not present in other established models of type 2 diabetes such as nSTZ and GK rats *(7–9)*, makes this novel diabetic syndrome particularly suitable for pharmacologic studies of new potential antidiabetic compounds. Indeed, STZ-NA-diabetic rats are being increasingly utilized in pharmacologic research (e.g., *10,11*).

The insulinotropic effects of tungstate treatments of different duration were explored in vivo by intravenous glucose tolerance tests (IVGTTs) and in vitro by using isolated perfused pancreas preparations when the in vivo results were indicative of an enhancement in insulin secretory function. Furthermore, taking into account that tungstate might exert

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Table 1
Effects of 2-wk Tungstate Treatment on Food, Fluid, and Tungstate Intake and Body Weight Gain of Control and Diabetic Rats[a]

	Fluid intake (mL/d)	Food intake (g/d)	Tungstate intake (mg/d)	Body weight gain (g/d)
Control rats				
Untreated (n = 14)	26.3 ± 0.5	22.1 ± 0.2	—	1.8 ± 0.3
Treated (n = 15)	25.5 ± 0.4	21.8 ± 0.2	50.9 ± 0.7	1.6 ± 0.1
Diabetic rats				
Untreated (n = 15)	27.0 ± 0.4	21.1 ± 0.2	—	1.4 ± 0.2
Treated (n = 18)	27.5 ± 0.3[b]	21.3 ± 0.1	55.1 ± 0.5[b]	1.5 ± 0.2

[a] Data are means ± SEM of the number of animals indicated in parentheses, pooled from two separate experiments.
[b] $p < 0.01$ vs treated control rats (student's t-test).

an insulin-like action on peripheral tissues *(1,12,13)*, we measured insulin-stimulated lipogenesis in isolated adipocytes and muscle glycogen levels as an indirect index of muscle glucose metabolism. Finally, hepatic glucose metabolism was characterized for the first time in STZ-NA-diabetic animals, and the action of tungstate on such metabolism was explored as well.

Results

Effects of 2-wk Tungstate Treatment on Food and Fluid Intake and on Body Weight

Oral administration of tungstate for 2 wk did not significantly affect food and fluid intake in either healthy or diabetic rats (Table 1). Since in tungstate-treated diabetic animals liquid consumption was slightly higher than in controls, the daily intake of tungstate was correspondently slightly higher. The daily body weight gain of either group was not significantly modified by tungstate treatment.

Effects of Tungstate Treatment on Basal Glycemia, Insulinemia, and Glucose Tolerance

Two weeks after the administration of STZ and NA, diabetic rats showed a mild hyperglycemia and a slightly reduced insulinemia (plasma glucose: 112 ± 5 and 137 ± 3 mg/dL, for control [n = 9] and diabetic [n = 27] rats, respectively, $p < 0.01$; plasma insulin: 3.8 ± 0.5 and 2.8 ± 0.2 ng/mL, for control and diabetic rats, respectively, $p < 0.05$). Tungstate treatment for 2 wk had no effect on basal glycemia and insulinemia in both control and diabetic animals (see Fig. 1 and Table 2). However, this treatment was able to reduce glucose intolerance in diabetic rats, as shown by the time course of glycemic variations after an iv glucose load (Fig. 1A), apparently through a substantial increase in postloading plasma insulin levels as compared with untreated diabetic animals (Fig. 1B). In tungstate-treated diabetic rats, the improvement in glucose tolerance was assessed by the significant increase in K coefficient as compared with untreated diabetic rats (1.71 ± 0.20 vs 1.08 ± 0.10%, $p < 0.02$; K of controls was 2.60 ± 0.10%). The enhancement in the insulin response was confirmed by calculating the postloading integrated incremental insulin values (ΔI) and by the ratio $\Delta I/\Delta G$, which were both significantly improved with respect to untreated animals (ΔI was 1.22 ± 0.10 ng/[mL·min] in treated vs 0.32 ± 0.05 ng/[mL·min] in untreated diabetic rats, $p < 0.01$; ΔI of controls was 2.99 ± 0.15 ng/[mL·min]).

On the basis of these results, we prolonged tungstate treatment in another cohort of rats, in an attempt to obtain a more substantial improvement in glucose metabolism. IVGTTs performed after 2 wk of treatment substantially confirmed the previous data (not shown), but no significant differences in glycemic and insulinemic IVGTT profiles were unexpectedly observed between treated and untreated animals after 30 d of treatment (Fig. 1C,D). Nevertheless, basal glycemia was slightly lower (see Table 2) and postloading insulin levels were consistently higher in treated than in untreated diabetic rats (ΔI was 1.45 ± 0.58 and 0.74 ± 0.23, respectively, NS; ΔI of controls was 2.12 ± 0.46). Thus, tungstate administration was further prolonged in the same animals (always at the dose of 2 mg/mL of drinking water). IVGTTs performed after 60 and 90 d of treatment showed that the effects of tungstate on glucose tolerance and postloading insulin levels were not further modified (at 60 d, in treated diabetic rats, K coefficient and ΔI were 1.11 ± 0.28% and 1.17 ± 0.24 ng/[mL·min], respectively, vs 1.43 ± 0.34% and 0.73 ± 0.14 ng/[mL·min] in untreated diabetics [NS] and vs 3.10 ± 0.33% and 2.33 ± 0.55 ng/[mL·min] in controls; at 90 d, similar values were obtained for such parameters). The tendency toward a reduction in basal glycemia was also confirmed (Table 2), achieving statistical significance at 60 d of treatment, as evaluated by student's t-test. However, it should be stressed that the differences

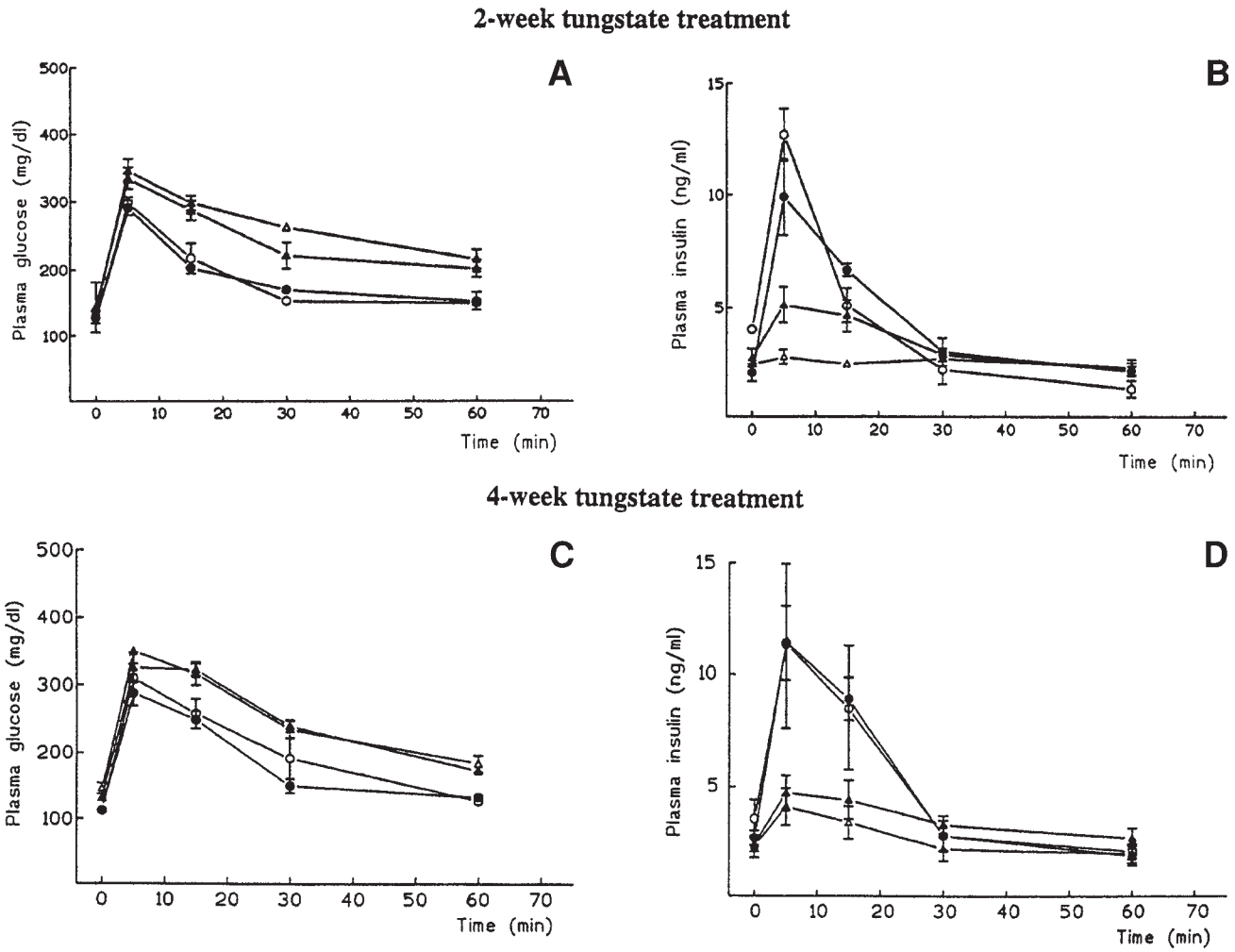

Fig. 1. Plasma glucose (**A,C**) and insulin (**B,D**) levels during IVGTT (0.75 g/kg of body wt) performed in nonfasting animals after 2 (top) or 4 (bottom) wk of oral tungstate administration (2 mg/mL of drinking water). Data are means ± SEM. (○) Untreated controls ($n = 4$); (●) tungstate-treated controls ($n = 4$–7); (△) untreated diabetics ($n = 5$); (▲) tungstate-treated diabetics ($n = 7$–9).

Table 2
Plasma Glucose Levels of Nonfasting Control and Diabetic Rats Following Oral Tungstate Treatment of Various Durations[a]

	Plasma Glucose (mg/dL)			
	15 d	30 d	60 d	90 d
Control rats				
Untreated (C, $n = 5$)	126 ± 4.4	113 ± 4.1	123 ± 5.1	130 ± 4.5
Treated (CT, $n = 5$–7)	128 ± 6.4	113 ± 3.8	116 ± 7.7	116 ± 6.7
Diabetic rats				
Untreated (D, $n = 5$–6)	144 ± 3.8	146 ± 8.0	153 ± 10.0	170 ± 8.3
Treated (DT, $n = 7$–9)	138 ± 3.8	131 ± 2.9	124 ± 4.9	150 ± 8.4

[a]Data are means ± SEM of the number of animals indicated in parentheses. C, untreated controls; D, untreated diabetics; CT, tungstate-treated controls; DT, tungstate-treated diabetics. Statistical analysis (ANOVA) showed that the differences among groups of animals ($F = 14.7$) and days of treatment ($F = 11.0$) were significant ($p < 0.01$ for both), as was the interaction ($F = 3.64$, $p < 0.05$). By the Tukey test, the differences between C and D, C and DT, CT and D, and CT and DT were significant ($p < 0.05$ at least), whereas the differences between D and DT were not.

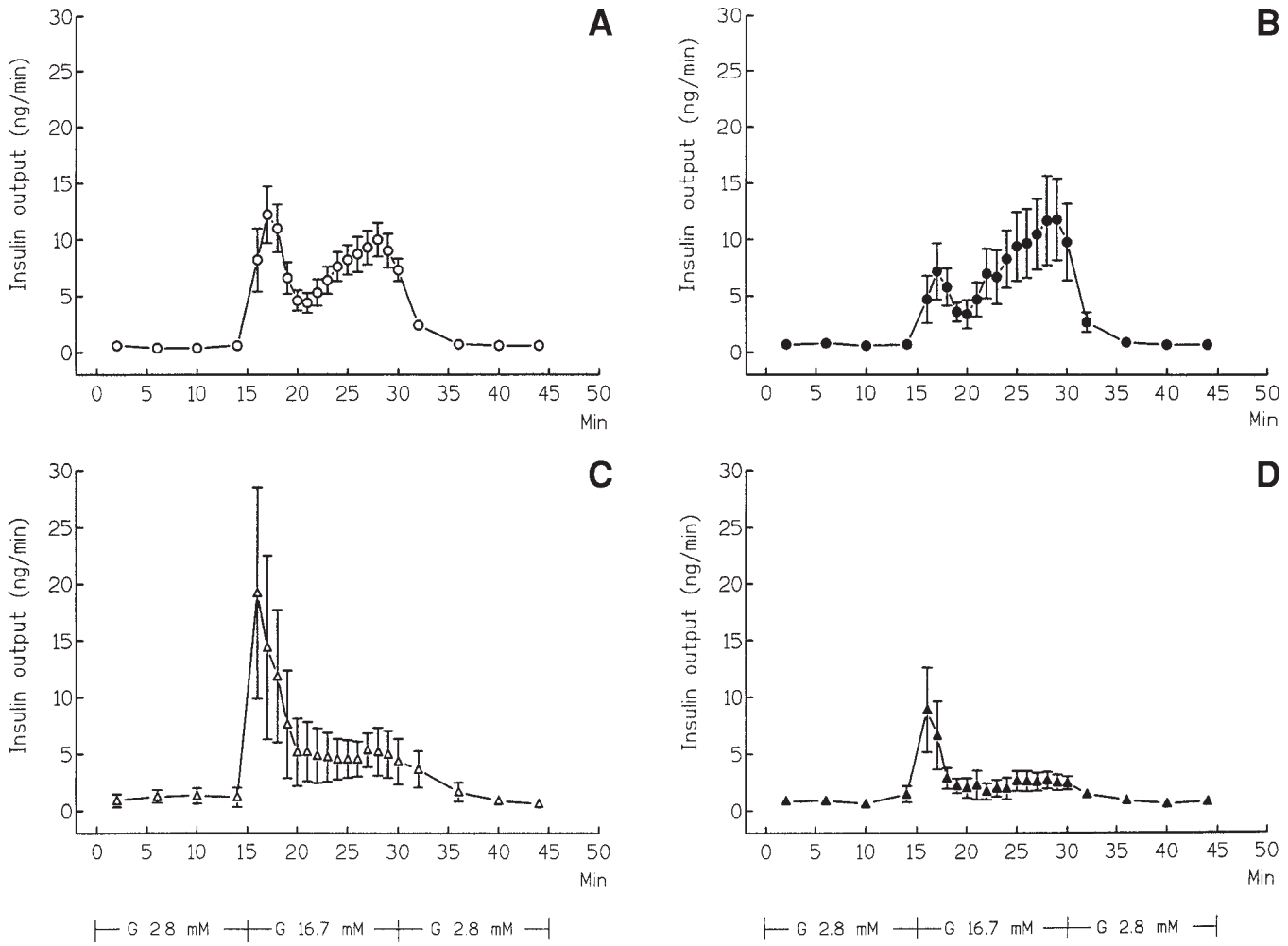

Fig. 2. Glucose (G)-stimulated insulin secretion from isolated perfused pancreas of (**A**) untreated control (○), (**B**) tungstate-treated control (●), (**C**) untreated diabetic (△), and (**D**) tungstate-treated diabetic (▲) rats after 2 wk of tungstate administration. Data are means ± SEM of eight and five observations for each group of control and diabetic rats, respectively.

in glycemic values between untreated and treated diabetic groups were not statistically significant, as assessed by analysis of variance (ANOVA) (see Table 2).

Two-Week Tungstate Treatment

Effect on Insulin Output from Isolated Perfused Pancreas

The insulin output from the isolated perfused pancreas stimulated by a rise in glucose concentration from 2.8 to 11 mmol/L of glucose is shown in Fig. 2A–D. In healthy rats, the typical biphasic pattern of glucose-stimulated insulin release occurred (Fig. 2A) and was not substantially affected by tungstate treatment, the only difference being a slight, not significant, reduction in the first-phase peak (Fig. 2B). In untreated diabetic animals, the first phase of insulin secretion was preserved, whereas the second phase was markedly defective (Fig. 2C), as expected in this model (6). Tungstate treatment of diabetic animals did not correct this impaired insulin responsiveness, which even showed a trend to a further decline (Fig. 2D). Pancreatic insulin content, measured after the perfusion, was found moderately but not significantly increased in tungstate-treated vs untreated diabetic animals (101 ± 14 vs 74 ± 15 μg/g of pancreas, respectively, NS; insulin content of control pancreas was 187 ± 27 μg/g).

Effect on Muscle Glycogen

Glycogen content in both fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (S) muscles of this cohort of rats was unaffected by diabetes as well as by a 2-wk tungstate treatment (Table 3).

Three-Month Tungstate Treatment

Effect on Insulin Content of Diabetic Islets

The insulin content of islets isolated from diabetic rats was decreased by approx 40% with respect to control islets (38 ± 4.0 vs 59 ± 5.4 ng/islet, $p < 0.05$), as expected *(5)*, and was partially restored following administration of tungstate (49 ± 3.1 ng/islet, $p < 0.05$ vs untreated diabetic islets).

Table 3
Glycogen Levels in EDL and S Muscles after 2 wk of Oral Tungstate Administration in Control and Diabetic Rats[a]

	Glycogen content (mg/g muscle)	
	EDL	S
Control rats		
Untreated (n = 4)	3.91 ± 0.33	4.13 ± 0.63
Treated (n = 4)	3.93 ± 0.26	3.00 ± 0.28
Diabetic rats		
Untreated (n = 6)	3.34 ± 0.44	4.47 ± 0.32
Treated (n = 6)	3.96 ± 0.14	3.80 ± 0.12

[a]Data are means ± SEM of the number of animals indicated in parentheses.

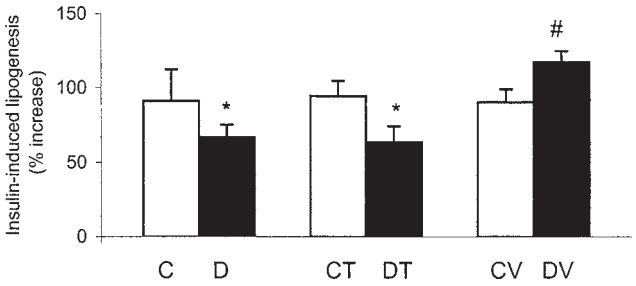

Fig. 3. Insulin-stimulated lipogenesis in adipocytes isolated from control and diabetic rats after 3 mo of tungstate or vanadyl sulfate administration. Results are expressed for each group as the percentage of increase over its own basal value (i.e., without insulin). Data are means ± SEM of four to six observations for each group. *$p < 0.05$ vs corresponding controls; #$p < 0.05$ vs untreated diabetics (student's t-test). C, untreated controls; D, untreated diabetics; CT, tungstate-treated controls; DT, tungstate-treated diabetics; CV, vanadyl sulfate–treated controls; DV, vanadyl sulfate–treated diabetics.

Nevertheless, the impaired insulin responsiveness of these islets to glucose, as explored in vitro during static incubations, was not modified by the tungstate treatment (data not shown).

Effect on Adipocyte Metabolism

Figure 3 summarizes the results of insulin-stimulated lipogenesis in isolated adipocytes. In untreated diabetic rats, insulin-induced lipogenesis was significantly impaired compared with controls and was not improved on tungstate administration for 3 mo. Conversely, a 3-month oral treatment with vanadyl sulfate (0.5 mg/mL of drinking water), performed in parallel in a group of diabetic rats of the same cohort, was able to fully prevent the impairment of insulin action in adipose tissue. In a separate experiment, a 30-d tungstate treatment was likewise unable to correct the defective insulin-induced lipogenesis in adipocytes from rats made diabetic 2 mo earlier (data not shown).

Table 4
Various Hepatic Metabolites and Enzymatic Activities in STZ-NA-Diabetic Rats and Effects of 15-d Oral Tungstate Treatment[a]

	Control rats (n = 8–10)	Diabetic rats (n = 8–10)	Tungstate-treated diabetic rats (n = 4–5)
Glycogen (mg/g liver)	34.9 ± 2.1	36.6 ± 2.1	35.8 ± 3.2
Glucose 6-phosphate (nmol/g liver)	487 ± 65	556 ± 52	512 ± 73
Glycogen synthase (U/g liver)	0.35 ± 0.08	0.34 ± 0.07	ND
Glycogen phosphorylase *a* (U/g liver)	23.7 ± 3.7	25.0 ± 2.4	ND
Glucokinase (U/g liver)	3.76 ± 0.36	3.22 ± 0.36	3.53 ± 0.16
Pyruvate kinase			
Total activity (U/g liver)	19.8 ± 3.5	17.5 ± 1.7	16.7 ± 1.5
Activity ratio ($V_{0.15}/V$)	0.19 ± 0.01	0.19 ± 0.02	0.17 ± 0.01

[a]Data are means ± SEM of the number of animals indicated in parentheses. For control and untreated diabetic rats, data were pooled from two separate experiments, except for pyruvate kinase, which was assayed in a single cohort of animals (n = 5). ND, not determined.

Liver Glucose Metabolism and Effect of Tungstate

A number of metabolites and enzymes that are among the key regulators of hepatic glucose metabolism were determined, for the first time, in STZ-NA-diabetic rats, as shown in Table 4. In the liver of diabetic animals, no significant changes in glycogen and glucose-6-phosphate levels as well as in glucokinase, pyruvate kinase, glycogen synthase, and glycogen phosphorylase *a* activities were observed with respect to control rats. When some of such metabolites or enzymes were measured in the liver of diabetic rats submitted to a 15-d tungstate treatment, no significant difference was found with respect to untreated diabetic animals (Table 4).

Discussion

The aim of our study was to evaluate the effect of oral tungstate administration in a new experimental diabetic syndrome, obtained in adult rats by the combined administration of STZ and NA *(6)*, that shares a number of features with human type 2 diabetes such as insulin responsiveness to sulfonylureas. We used a sodium tungstate dosage (2 mg/mL in drinking water) previously shown to have hypoglycemizing effects in other experimental models of diabetes and to be devoid of toxic effects *(1–4)*, probably owing to

the high bioavailability of this compound in rats *(14)*. In this regard, we observed no significant change in food and water intake and in body weight gain in tungstate-treated compared with untreated rats.

Our results show that oral tungstate administration for 2 wk had no effect on the basal moderate hyperglycemia of STZ-NA-diabetic rats but was able to partially correct their glucose intolerance. This latter effect was most likely mediated by a substantial increase in postloading insulin secretion, resulting in a more rapid glucose removal from blood. Quite surprisingly, we observed that the beneficial action of tungstate on glucose tolerance in diabetic rats was only transient, being no more detectable in IVGTT experiments performed after 30, 60, and 90 d of treatment. However, note that in the diabetic groups submitted to prolonged tungstate administration, postloading insulin secretion was not very different from that observed after 15 d of treatment, whereas there was a complete loss of the beneficial effect on glucose tolerance (e.g., see Fig. 1B,D). This probably means that alterations in peripheral insulin sensitivity, rather than further defects in insulin production, have been developing in these groups of animals and are not amendable by tungstate treatment. The slight but consistent reduction in basal hyperglycemia observed in our diabetic rats on prolonged tungstate administration should be emphasized, although this reduction did not achieve statistical significance.

After 15 d of tungstate administration, we explored the in vitro secretory response of the perfused pancreas of tungstate-treated diabetic rats and found that, at variance with the in vivo observations during an IVGTT, no increase in glucose-stimulated insulin secretion occurred with respect to untreated animals, despite some increase in pancreatic insulin content, found at the end of the perfusion. This discrepancy might perhaps be attributed to the lack of important modulatory signals in vitro, such as parasympathetic nervous activity, which is abolished by the subdiaphragmatical vagotomy performed during surgical preparation of the perfused pancreas.

In pancreatic islets isolated from diabetic rats submitted to a 3-mo tungstate treatment, although no functional improvement could be detected (data not shown), the insulin content was significantly higher than that found in the islets of untreated diabetic animals. A similar increase in islet insulin content associated with the absence of functional improvement has been observed in rat islets exposed to tungstate in vitro *(15)*. The increase in islet insulin content after an in vivo tungstate treatment, already observed in nSTZ-diabetic rats *(2)*, might be attributed to an increase in β-cell mass or to an increase in the expression of the insulin gene with a consequent increase in hormone biosynthesis *(2)*, but the definite mechanism remains to be elucidated.

To explore the effect of tungstate on possible alterations in peripheral insulin sensitivity in our model of type 2 diabetes, we considered two major targets of insulin action: skeletal muscle and adipose tissue. In the muscle, after 15 d of tungstate administration, glycogen levels were unaffected by tungstate treatment, thereby confirming previous observations in STZ-diabetic rats *(1)*. In adipose tissue, our data indicate that in STZ-NA-diabetic rats, insulin resistance was apparent at both 2 (not shown) and 3.5 mo after diabetes induction, as indicated by the decreased capability of insulin to stimulate lipogenesis in the isolated adipocytes. This defect was corrected by vanadyl sulfate but not tungstate treatment. A similar difference in the effects of tungstate and vanadate on adipocyte metabolism has been recently reported *(16)*, being the antilipolytic activity of these compounds relevant for vanadate and negligible for tungstate. Thus, our results confirm the observation that tungstate and vanadate, although sharing some metabolic effects, may influence different intracellular molecular pathways (see ref. *3*).

On the whole, our results indicate that in the STZ-NA model of type 2 diabetes, the effects of tungstate administration were modest and transient, different from what has been observed in other experimental models of diabetes. This discrepancy is intriguing, but we suggest that it could be interpreted on the basis of the fact that the beneficial effect of tungstate might largely be owing to correction of the abnormalities in liver metabolism that occur in STZ-diabetic *(1,4)*, nSTZ-diabetic *(2)*, and Zucker *(3)* rats but are not present in STZ-NA-diabetic animals, as we report here. In fact, it is likely that in STZ- and nSTZ-diabetic rats, the amelioration of hepatic glucose metabolism on tungstate treatment may result in an increase in glycolytic flux and a reduction in hepatic glucose production, which might be crucial for the restoration of glucose homeostasis. Furthermore, it should be stressed that tungstate is a potent competitive inhibitor of glucose-6-phosphatase *(17)* and that such a property might play an important role in the normalization of glycemia in STZ-diabetic rats, in which an increase in liver glucose-6-phosphate levels *(1,2)* and an enhancement of glucose-6-phosphatase activity *(18)* have been reported. Taking into account these considerations, the failure of tungstate to permanently correct glucose metabolism in the STZ-NA model of diabetes could well be attributed to the absence of any relevant hepatic alteration in this diabetic syndrome, including glucose-6-phosphate levels and key enzymes in the regulation of liver carbohydrate metabolism.

As a final comment, our results seem to indicate that the antidiabetic action of tungstate could be less ample than previously supposed. In particular, no insulin-like effect in the muscle and adipose tissue has been observed. From comparison of the results obtained in different models of diabetes, it appears that the major role of tungstate could be correction of hepatic alterations, with the possible contribution of a stimulating effect on β-cell proliferation and/or insulin biosynthesis, which, however, should be confirmed by further investigation.

Materials and Methods

Animals

Experiments were performed in male Wistar rats 2.5 mo of age, weighing 220–230 g. Animals had free access to food (standard Randoin Causeret diet in pellets), were kept at 24 to 25ºC, and were subjected to a 12:12 h photoperiod. The National Institute of Health principles of laboratory animal care were followed as well as the recommendation of Italian law for the use of experimental animals (DL n. 116/1992), including the approval of experimental protocols by a review committee at the University of Pisa.

Treatments

NA (270 mg/kg of body wt), dissolved in saline, was injected intraperitoneally 15 min before iv administration of 60 mg/kg of STZ (Sigma, St. Louis, MO), dissolved in buffer citrate (pH 4.5) immediately before use. Controls received the vehicles of both substances. Sodium tungstate (2 mg/mL of drinking water) was given to subgroups of both control and diabetic rats starting 2 wk after diabetes was induced. Tungstate-containing water was replenished every other day. Untreated animals received tap water. The treatment was carried out for variable periods of time (15, 30, 60, and 90 d). During the experimental period, animal body weights as well as fluid and food intakes were measured every other day.

Intravenous Glucose Tolerance Test

Between 9 and 10 AM, glucose (0.75 g/kg of body wt) was given as a 30% solution through the tail vein of nonfasting conscious animals. Blood samples were collected sequentially from the tail vein before and 5, 15, 30, and 60 min after the injection. After centrifugation at 4ºC, plasma was separated and then used immediately for glucose determination, with the remainder kept at –20ºC for insulin measurement by radioimmunoassay (RIA). Glucose tolerance was quantitated using two parameters: ΔG (integrated increase in glycemia over baseline over a period of 60 min after the iv glucose load), and K coefficient (glucose disappearance rate between 5 and 30 min after glucose administration). Insulin secretion during the IVGTT was quantitated as the incremental insulin values integrated over 60 min after the load (ΔI); the insulinogenic index ($\Delta I/\Delta G$) was also calculated.

Isolated Perfused Pancreas Technique

Pancreas was isolated and perfused according to the method of Penhos et al. *(19)*, as modified by Gerber et al. *(20)*. Pancreas was perfused with a modified Krebs-Ringer bicarbonate buffer (pH 7.4) with 4% dextran T40 and 0.25% bovine serum albumin (BSA). The perfusing buffer was continuously gassed with a mixture of 95% O_2 and 5% CO_2, pH 7.4. Flow rate was kept constant at 3 mL/min. After 30 min of equilibration, pancreatic effluent was collected in 1-min aliquots from a catheter inserted into the portal vein and stored at –20ºC until insulin RIA. The perfusion protocol consisted of three consecutive periods of 15 min each with the following conditions: 2.8 m*M*, 16.7 m*M*, and 2.8 m*M* glucose. The insulin output was calculated from the insulin concentration in the perfusate (nanograms/milliliter) multiplied by the flow rate. At the end of the perfusion, most of the pancreas was removed, weighed, and homogenized in cold acidified ethanol (150:47:3 [v/v/v], absolute ethanol:H_2O: concentrated HCl) for extraction of insulin, as detailed elsewhere *(21)*.

Muscle Glycogen

The fast-twitch EDL and the slow-twitch S muscles were removed as quickly as possible, immediately frozen with metal clamps precooled in liquid nitrogen, and stored at –70ºC until processed. Muscles were hydrolyzed in boiling 30% KOH for 30 min, and glycogen was precipitated twice with ethanol and then assayed by the anthrone method of Hassid and Abraham *(22)*.

Insulin-Stimulated Lipogenesis in Isolated Adipocytes

Adipocytes were prepared from epididymal fat pads pooled from two to three rats for each group and each experiment by the collagenase digestion procedure by Rodbell *(23)* with minor modifications. Krebs-Ringer-HEPES buffer (pH 7.4), containing 4% BSA and 5 m*M* glucose, was used in all the isolation and incubation steps. The ability of insulin to stimulate lipogenesis was assessed by monitoring the incorporation of [^{3}H]-glucose into total fat cell lipid, according to the method of Moody et al. *(24)*. Insulin-stimulated lipogenesis was expressed as a percentage of the basal value, obtained without addition of insulin.

Determination of Metabolites and Enzymes in Liver

Liver glycogen content was determined according to Chan and Exton *(25)*. Glucose-6-phosphate levels were measured in the neutralized perchloric extracts from frozen samples, as described previously *(26)*. Glucokinase, glycogen synthase, and glycogen phosphorylase *a* activities were determined as previously described *(2)*. Total pyruvate kinase activity and activity ratio ($V_{0.15}/V$, measured at 0.15 and 5 m*M* phosphoenolpyruvate, respectively) were determined as detailed elsewhere *(1)*.

Isolation of Islets

Pancreatic islets were isolated by the collagenase method using the procedure of pancreatic duct cannulation and purification by density gradient *(27)*. Batches of 8–10 freshly isolated islets, after several washes, were used either for static incubation experiments to assess their responsiveness to glucose or for extraction of insulin content by addition of 1 mL of cold acidified ethanol.

Glucose and Insulin Assays

Plasma glucose was measured by the glucose oxidase method using commercially available kits (Glucinet; Sclavo,

Italy). Insulin was measured by RIA according to Herbert et al. *(28)* using rat insulin as a standard. The sensitivity and the coefficient of variation of the assay were as follows: detection limit 0.13 ng/mL, intraassay variation of 3.2%, interassay variation of 9.8%.

Statistical Analyses

Data are given as means ± SEM. Statistical significance was evaluated by either student's *t*-test or ANOVA followed by Tukey test to assess two-by-two differences, as appropriate.

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