Effects of Tungstate, a New Potential Oral Antidiabetic Agent, in Zucker Diabetic Fatty Rats

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Tungstate was orally administered to 7.5-week-old male Zucker diabetic fatty (ZDF) rats that already showed moderate hyperglycemia (180 ± 16 mg/dl). The animals became normoglycemic for ~10 days. Then, glycemia started to rise again, although it did not reach the initial values until day 24, when levels stabilized at ~200 mg/dl for the duration of the experiment. Untreated ZDF rats showed steadily increased blood glucose levels between 7.5 and 10 weeks of age, when they reached a maximum value of 450 ± 19 mg/dl, which was maintained throughout the experiment. In addition, tolerance to intraperitoneal glucose load improved in treated diabetic rats. Serum levels of triglycerides were elevated in untreated diabetic rats compared with their lean counterparts (ZLC). In the liver of diabetic animals, glucokinase (GK), glycogen phosphorylase *a* (GP*a*), liver-pyruvate kinase (L-PK), and fatty acid synthase (FAS) activities decreased by 81, 30, 54, and 35%, respectively, whereas phosphoenolpyruvate carboxykinase (PEPCK) levels increased by 240%. Intracellular glucose-6-phosphate (G6P) decreased by 40%, whereas glycogen levels remained unaffected. Tungstate treatment of these rats induced a 42% decrease in serum levels of triglycerides and normalized hepatic G6P concentrations, GP*a* activity, and PEPCK levels. GK activity in treated diabetic rats increased to 50% of the values of untreated ZLC rats. L-PK and FAS activity increased to higher values than those in untreated lean rats (1.7-fold L-PK and 2.4-fold FAS). Hepatic glycogen levels were 55% higher than those in untreated diabetic and healthy rats. Tungstate treatment did not significantly change the phosphotyrosine protein profile of primary cultured hepatocytes from diabetic animals. These data suggest that tungstate administration to ZDF rats causes a considerable reduction of glycemia, mainly through a partial restoration of hepatic glucose metabolism and a decrease in lipotoxicity. *Diabetes* 50:131–138, 2001

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ALT, alanine transaminase; AST, aspartate transaminase; BMOV, bis(maltolato)oxovanadium(IV); FAS, fatty acid synthase; G6P, glucose-6-phosphate; GK, glucokinase; GP*a*, glycogen phosphorylase *a*; GS, glycogen synthase; HK, hexokinase; L-PK, liver-pyruvate kinase; nSTZ, neonatally injected STZ; PEPCK, phosphoenolpyruvate carboxykinase; STZ, streptozotocin.

Previous results from our group showed that oral administration of sodium tungstate to rats made insulin deficient and diabetic by streptozotocin (STZ) injection caused a dramatic decrease in their blood glucose concentration (1,2). Two animal models of diabetes, STZ (corresponding to type 1 diabetes model) and neonatally injected STZ (nSTZ) rats (resembling a type 2 diabetes model) were tested. In the first case, the main action of tungstate appears to be the restoration of the hepatic glucose metabolism by increasing the capacity of the liver to utilize glucose through glycolysis and glycogenesis, and to decrease its potential for glucose output (1). However, this does not appear to be the case in rats with diabetes induced by nSTZ; in these rats, the normoglycemic effect of tungstate cannot be attributed to the small changes observed in hepatic glucose metabolism. Moreover, the main action of tungstate appears to be correlated with an increase in insulin content and β-cell mass, which leads to an improvement in the ability of β-cells to respond to glucose (2). Although the results on the normoglycemic capacity of tungstate obtained with the induced models are very encouraging, they are hampered by the limitations associated with STZ-induced diabetes (3,4).

Among the different genetic animal models for type 2 diabetes, ZDF rats are the most widely used because the development of the disease in males shares many features with that in humans (5). It is considered the closest available rat model to human type 2 diabetes associated with obesity. Homozygous ZDF *fa/fa* males are obese and develop progressive insulin resistance and glucose intolerance at an early age (between 3–8 weeks of age), and they become overtly diabetic and hyperinsulinemic at 8–10 weeks (5,6). The lean littermates (ZLC), which are heterozygous +/*fa* or homozygous +/+, develop neither insulin resistance nor diabetes (7). ZDF rats have been used to test new therapeutic agents for type 2 diabetes (8–15).

Our aim was to test whether tungstate improves glucose homeostasis in these genetically obese and diabetic rats. Oral administration of tungstate for 2 months to male ZDF rats initially reverted hyperglycemia. Although this was not a permanent effect, these treated animals never reached the very high glucose levels observed in untreated rats. In addition, the administration of tungstate decreased the elevated serum triglyceride levels. These results show that tungstate is also an effective antidiabetic agent in this type 2 diabetes genetic model.

RESEARCH DESIGN AND METHODS

Materials. Sodium tungstate was from Carlo Erba (Milan, Italy). Enzymes and biochemical reagents were either from Boehringer Mannheim (Mannheim, Germany) or Sigma (St. Louis, MO), unless otherwise indicated. All other chemicals were of analytical grade.

Animals. Male Zucker diabetic fatty (ZDF/GmiTM*fa/fa*) rats were 7.5 weeks old (onset of diabetes) at the beginning of the treatments. Lean age-matched Zucker control rats (ZLC/GmiTM *+/fa* or +/+) were used. All of the animals used in this study were purchased from Genetic Models (Indianapolis, IN). Animals were kept under a constant 12-h light-dark cycle and were allowed to eat (Purina 5008) and drink ad libitum. The experiments were performed in two sets for 60 days. All institutional guidelines for the care and use of animals were followed.

At the beginning of the experiment, both diabetic and healthy animals were divided into two groups. The first group (untreated) received drinking water, whereas the second group (treated) was given a solution of 2 mg/ml sodium tungstate in distilled water. The treatment was carried out for 2 months. During this period, the fluid and food intakes were measured between 10:00 and 12:00 AM., and the body weight and blood glucose levels (Reflotron; Boehringer Mannheim) were measured every 2 days.

At the end of the treatment, rats were killed by decapitation. Blood was immediately collected to measure serum parameters, and additional blood samples were collected on EDTA. After centrifugation at 1,000*g* for 15 min at 4°C, plasma was removed and stored at –20°C. Plasma insulin was measured by radioimmunoassay (CIS; Bioternational, Gif-Sur-Yvette, France). Livers were excised and sliced; fragments were either used immediately to measure enzyme activity or were rapidly frozen in liquid N_2 for later processing.

Glucose tolerance testing. After an overnight fast, rats received 2 g/kg glucose intraperitoneally. Glucose concentrations were measured before and at 15, 30, 60, 90, and 120 min after glucose administration. These studies were performed on animals anesthetized with pentobarbital, which was administrated intraperitoneally (60 mg/kg body wt).

Analytical procedures in serum samples. Serum alanine transaminase (ALT), aspartate transaminase (AST), urea, triglyceride, and cholesterol levels were measured spectrophotometrically by standard techniques adapted to a Dax 72 analyzer (Bayer Diagnostics, Leverkusen, Germany).

Enzyme activity assay. Glycogen synthase (GS) and glycogen phosphorylase *a* (GP*a*) activities were determined in fresh liver samples homogenized in 10 vol 10 mmol/l Tris-HCl (pH 7.4) containing 150 mmol/l KF, 15 mmol/l EDTA, 600 mmol/l sucrose, 50 mmol/l 2-mercaptoethanol, 10 µg/ml leupeptin, 1 mmol/l benzamidine, and 1 mmol/l phenylmethylsulfonyl fluoride . The homogenates were centrifuged at 10,000*g* for 15 min at 4°C, and the resulting supernatants were used for determinations. The GS activity ratio (–glucose-6-phosphate [G6P]/+G6P) and total activity were measured as previously described (16). GP*a* activity was determined as described by Gilboe et al. (17). Glucokinase (GK) and hexokinase (HK) activities were measured in fresh liver samples homogenized in 10 vol ice-cold 50 mmol/l Tris-HCl buffer (pH 7.4) containing 1 mmol/l EDTA, 100 mmol/l KCl, 300 mmol/l sucrose, and 10 mmol/l 2-mercaptoethanol. The homogenates were centrifuged at 10,000*g* for 15 min at 4°C. The GK and HK activities of the supernatants were determined as previously described (18). Liver pyruvate kinase (L-PK) activity was measured spectrophotometrically. Frozen liver samples were homogenized in 10 vol ice-cold buffer solution at pH 7.4 with 50 mmol/l glycilglycine, 15 mmol/l EDTA, 100 mmol/l KF, and 5 mmol/l potassium phosphate. The homogenates were centrifuged at 10,000*g* for 15 min at 4°C, and total L-PK activity and activity ratio ($V_{0.15}/V$, measured at 0.15 and 5 mmol/l phosphoenolpyruvate, respectively) were determined in the supernatants as described (19). FAS activity was measured spectrophotometrically at 25°C. Frozen liver samples were homogenized in 10 vol ice-cold buffer solution of 100 mmol/l KH_2PO_4, pH 7.0, and 0.1 mmol/l EDTA. The homogenates were centrifuged at 10,000*g* for 15 min at 4°C, and FAS activity was determined in the supernatants as previously described (20).

Metabolite determinations. Liver glycogen content was determined as described (21). Frozen liver samples were homogenized with 10 vol ice-cold 30% (wt/vol) KOH and boiled at 100°C for 15 min. Glycogen was determined after ethanol precipitation. The intracellular concentrations of G6P were measured by spectrophotometric assays (22).

Hepatocyte isolation and culture and tyrosine phosphorylation determination. ZDF rat hepatocytes were isolated from 24 h–fasted animals by collagenase perfusion and were cultured as previously described (23). Sodium tungstate, sodium orthovanadate, and insulin treatments were then performed as described in Fig. 7. After treatment, cells were immediately lysed with boiling 10 mmol/l Tris-HCl (pH 7.4), 1% SDS, and 1 mmol/l sodium orthovanadate. The protein concentration was determined using the BCA Protein assay kit (Pierce, Rockford, IL).

Proteins were separated by 5–15% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Proteins were then immunoblotted with mouse anti-phosphotyrosine monoclonal antibody PY20 (Transduction Labs, San Diego, CA); the immunoblots were developed using an enhanced chemiluminescence detection system (Amersham, Amersham, U.K.), as recommended by the manufacturer.

PEPCK Western blot analysis. Frozen liver samples were thawed, homogenized in 10 vol homogenization buffer (10 mmol/l Tris-HCl, pH7.4, 150 mmol/l NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 1 mmol/l phenylmethyl sulfonylfluoride), and centrifuged at 14,000*g* for 15 min at 4°C. Pellets were discarded and protein concentration of the supernatants was determined as described by Bradford (24).

Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes, which were processed for immunoblotting with a sheep polyclonal antibody to rat liver phosphoenolpyruvate carboxykinase (PEPCK) (provided by Dr. Daryl Granner, Vanderbilt University) (25) and developed as described above.

Pancreatic islet isolation and analysis. The pancreatic islets were isolated by the collagenase method (26). Islets were separated from the remaining exocrine tissue by handpicking under a stereomicroscope. Batches of eight islets were incubated in a shaking water bath for 90 min at 37°C in 1.0 ml bicarbonate-buffered medium containing bovine serum albumin (5.0 mg/ml) and D-glucose at 2.8 or 16.7 mmol/l. For the first 10 min of incubation, the vials containing the incubation medium with the islets were gassed with O_2:CO_2 (95:5). At the end of the incubation period, the supernatants were stored at –20°C until assayed for insulin by radioimmunoassay. Insulin content was measured in these islets after ultrasonic disruption at 4°C in 0.5 ml acid-alcohol solution (75% [vol/vol]) ethanol, 23.5% bidistilled water, and 1.5% (vol/vol) 10 mol/l HCl) and centrifugation at 1,500*g* for 10 min at 4°C. The resulting supernatants were kept at –20°C until insulin assay by radioimmunoassay.

Statistical analysis. Results were analyzed for significance by analysis of variance and unpaired Student's *t* test. $P < 0.01$ was considered significant.

RESULTS

Variations in glycemia attributable to tungstate administration. Untreated ZDF rats, which were 7.5 weeks old at the beginning of the experiment, showed mild hyperglycemia (180 ± 16 mg/dl) that slowly progressed, reaching levels of 450 mg/dl after 20 days remaining constant throughout the experiment, as previously described (5). In contrast, in untreated ZLC rats, glycemia levels were ~80 mg/dl during the experiment.

At the beginning of the treatment, tungstate normalized glycemia in diabetic animals to values similar to those of their lean littermates. The animals remained normoglycemic for about 10–12 days. Then, glycemia started to rise slowly again, reaching the initial concentrations by day 24, and it stabilized at an average value of 200 mg/dl, which was maintained constant throughout the treatment (Fig. 1*A*). Tungstate administration to ZLC animals did not exert any effect on glycemia.

Figure 1*B* shows that the untreated diabetic rats become hyperglycemic much faster than the tungstate-treated ones. At the moment when 100% of the untreated ZDF rats were already hyperglycemic, the percentage of treated diabetic animals with glycemia >140 mg/dl was only ~30%.

Next, a series of intraperitoneal glucose tolerance tests were performed. After an overnight fast, plasma glucose levels were lower in treated (140 ± 26 mg/dl) than in untreated diabetic animals (275 ± 28 mg/dl) (Fig. 2*A*). In addition, the area under the curve was smaller in treated diabetic animals compared with untreated ZDF rats (Fig. 2*B*).

Effects of tungstate treatment on physical status and blood parameters. Oral administration of tungstate to diabetic rats for 2 months did not exert any effect on growth rate compared with untreated diabetic animals. Tungstate treatment of ZLC rats slightly decreased the growth rate (Fig. 3).

To ascertain whether tungstate induced hepatic or renal damage, serum catalytic concentrations of ALT and AST and urea levels were measured. A twofold increase in ALT activity was observed in serum from diabetic rats compared with lean controls (Table 1). Tungstate administration to ZDF rats significantly decreased ALT activity, whereas it remained unvaried in treated ZLC animals. AST and urea levels did not change with treatment in either lean or diabetic animals, suggesting that tungstate does not exert any appreciable toxic effect on liver and kidney. Serum triglyceride concentrations were markedly increased in untreated ZDF rats compared

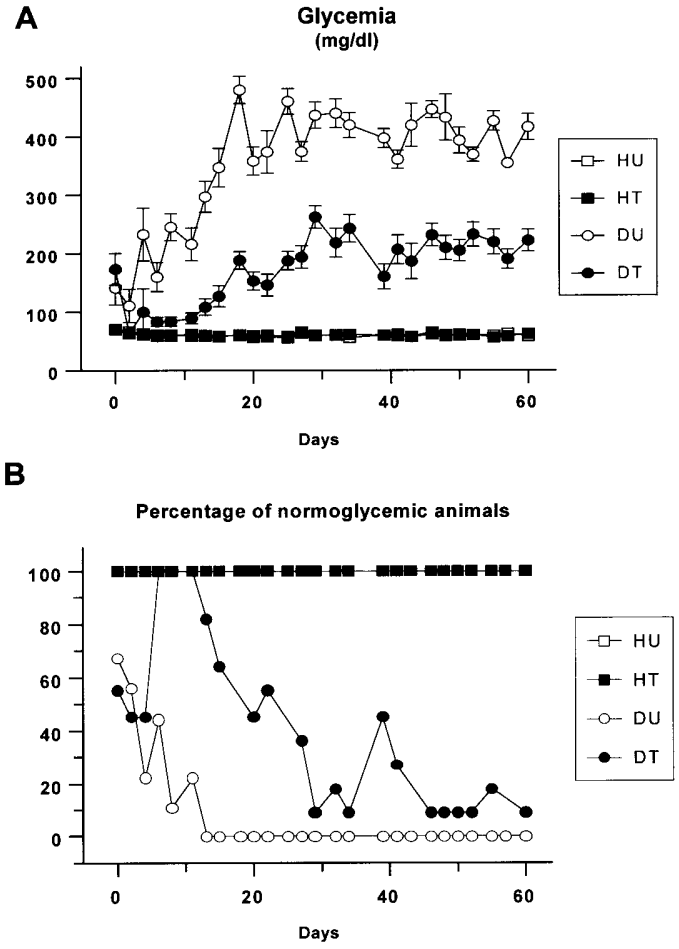


FIG. 1. ***A*****: Effects of tungstate treatment on blood glucose levels in vivo. Glycemia was measured from healthy untreated (HU), healthy treated (HT), diabetic untreated (DU), and diabetic treated (DT) animals. Values are means ± SE (*n* = 10).** ***B*****: Percentage of animals that remain normoglycemic throughout tungstate treatment (hyperglycemia [glucose] >140 mg/dl). Glycemia was measured in healthy untreated, healthy treated, diabetic untreated, and diabetic treated animals. Values are mean ± SE (*n* = 10).**

with ZLC untreated animals (Table 2). Tungstate treatment dramatically decreased the serum triglyceride concentration in diabetic rats, keeping it unchanged in the lean controls; however, it did not exert any effect on cholesterol levels. Tungstate treatment did not change serum insulin levels in either healthy or diabetic rats (Table 2).

Effects of tungstate treatment on hepatic glycogen and G6P. It was necessary to assess whether the reduced glycemia induced by tungstate in diabetic rats was partly attributable to an increase in glucose consumption by the liver, which would lead to glycogen accumulation. Hepatic glycogen levels were similar in both untreated ZDF and ZLC animals, indicating that liver glycogen deposition was not altered in ZDF rats in vivo (Fig. 4*A*). Tungstate treatment did not induce any modification of this parameter in lean rats, whereas in treated diabetic animals, hepatic glycogen levels increased 55% compared with both untreated ZLC and ZDF rats (Fig. 4*A*).

ZDF rats had a reduced G6P concentration, which was ~60% of lean rat values. Tungstate treatment of ZDF rats restored G6P to levels similar to those of the ZLC, whereas these G6P levels were not altered in lean animals (Fig. 4*A*).

Modifications of hepatic enzyme activities. The activities of the key enzymes in liver glucose, glycogen, and lipid metabolism were measured. The GK activity of ZDF rats was only ~20% that of the lean controls. However, HK activity was similar for both groups. Tungstate administration to diabetic animals increased GK activity to ~50% (a 2.5-fold increase) of the value of ZLC rats, but it did not modify HK activity. In lean animals, the treatment did not change either GK or HK activities (Fig. 4*B*).

GP*a* and GS are the two controlling enzymes of glycogen metabolism. ZDF rats showed a 30% decrease in GP*a* activity compared with untreated ZLC rats (Table 3). Tungstate treatment increased GP*a* activity to levels that did not significantly differ from those measured in lean animals. In treated ZLC rats, no significant modification was observed in GP*a* activity. Total GS activity was not significantly different among the four groups of animals assayed. Additionally, we did not observe any differences in the –G6P/+G6P activity ratio (Table 3).

L-PK is one of the rate-limiting steps in glycolysis. When the L-PK activity ratio was assayed, no significant differences

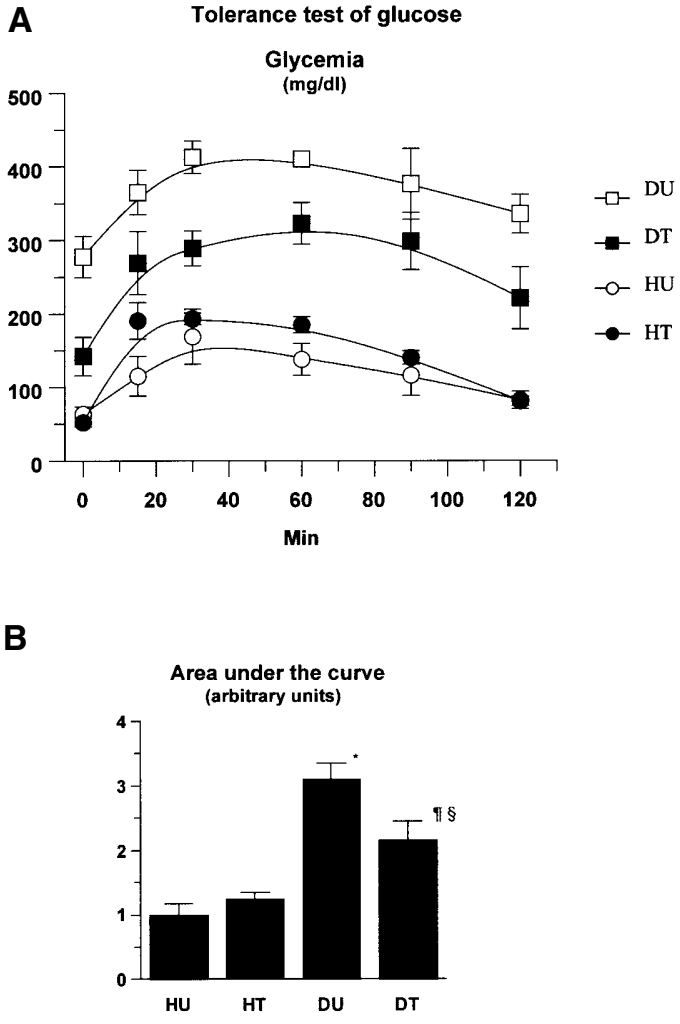


FIG. 2. Response to intraperitoneal administration of glucose (2 g/kg) (*A*) and area under the curve (*B*) in healthy untreated (HU), healthy treated (HT), diabetic untreated (DU), and diabetic treated (DT) animals. Values are means ± SE (*n* = 10). **P* < 0.001 compared with untreated healthy animals; ¶*P* < 0.05 compared with untreated and treated healthy animals; §*P* < 0.05 compared with untreated diabetic animals.

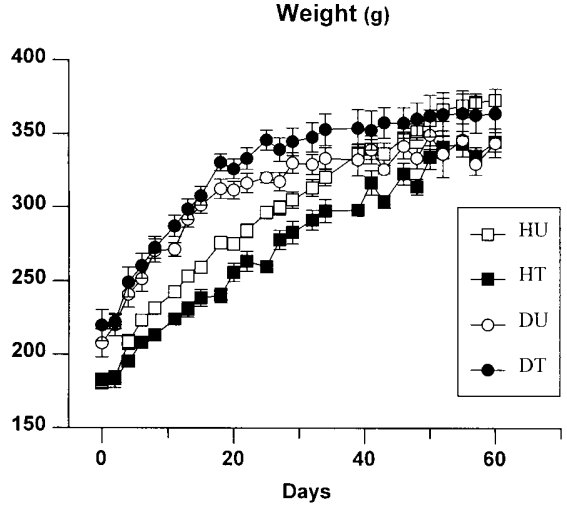


FIG. 3. Effects of tungstate treatment on body weight. The body weight was measured throughout tungstate treatment from healthy untreated (HU), healthy treated (HT), diabetic untreated (DU) and diabetic treated (DT) animals. Values are means ± SE (n = 10).

between the different groups of animals were found. However, total L-PK activity in diabetic rats was reduced to ~54% of the levels of lean animals. In contrast, in treated diabetic rats, total L-PK activity increased, reaching even higher levels than those found in untreated lean animals (1.7-fold). Tungstate treatment did not modify the activity in ZLC rats (Table 3).

Levels of PEPCK, the main regulatory enzyme of gluconeogenesis, were measured by Western blot. ZDF rats showed an increase in PEPCK levels (2.4-fold). Tungstate treatment of ZDF animals normalized PEPCK levels, whereas no effect was observed in lean animals (Fig. 5).

FAS is one of the rate-limiting enzymes of lipogenesis. Liver FAS activity was reduced to ~65% of the levels of lean animals. In contrast, in treated diabetic rats, FAS activity reached higher levels than those found in untreated lean animals (2-fold). Tungstate treatment did not modify this activity in ZLC rats (Table 3).

Effect of tungstate treatment on islet performance. The effect of tungstate on β-cell function in ZDF rats was tested. First, the β-cell response to glucose was analyzed. As expected, in both treated and untreated lean animals, there was a significant increase in insulin release when the glucose concentration was shifted from 2.8 to 16.7 mmol/l (Fig. 6*A*). In contrast, the increase in insulin release evoked by a 16.7 mmol/l glucose stimulus in pancreatic islets was totally abolished in untreated ZDF rats. When islets from treated diabetic rats were incubated with 2.8 mmol/l glucose, insulin secretion was lower than that in untreated lean or diabetic animals. When they were stimulated with a shift in glucose concentration, a small increase in insulin secretion was observed (Fig. 6*A*). Second, the insulin content in pancreatic islets was measured. Insulin content was markedly decreased in islets of diabetic animals (Fig. 6*B*), and tungstate administration further decreased it in ZDF but not in ZLC rats.

Effects of tungstate on protein tyrosine phosphorylation. Primary cultured hepatocytes from ZDF rats were incubated for short- (10–60 min) and long-term (>3 h) durations with either two sodium tungstate concentrations (100 µmol/l and 1 mmol/l) known to exert insulinomimetic effects on glycogen metabolism

TABLE 1
Clinic parameters of ZLC and ZDF rats

	n	ALT (U/l)	AST (U/l)	Urea (mg/dl)
Healthy Rats				
Untreated	10	68 ± 7	220 ± 14	46.4 ± 2.3
Treated	10	79 ± 5	208 ± 14	45.4 ± 2.4
Diabetic Rats				
Untreated	10	140 ± 16*	206 ± 24	51.1 ± 5.4
Treated	10	100 ± 8*†	183 ± 11	50.1 ± 2.3

Data are means ± SE. *P < 0.001 compared with untreated healthy animals; †P < 0.001 compared with untreated diabetic animals.

(data not shown) or sodium orthovanadate (50 µmol/l). Insulin was used as a positive control. The phosphotyrosine-containing proteins were analyzed by Western Blot as described in RESEARCH DESIGN AND METHODS. Short-term incubation (10–60 min) with both orthovanadate and tungstate did not significantly affect the phosphotyrosine pattern, including the insulin receptor subunits. However, during long-term incubations (6–12 h), while orthovanadate induced a general increase in phosphotyrosine-labeled bands, no clear-cut increase in phosphorylation was observed with tungstate (Fig. 7).

DISCUSSION

We have previously described that oral administration of sodium tungstate to rats normalizes glycemia in two animal models of diabetes, adult-injected STZ (1) and nSTZ rats (2). The former is a type 1 diabetes model (3,4,27,28), and the latter resembles type 2 diabetes (29,30). The major limitation of these models is that they only partially mimic the disease in humans. In particular, the nSTZ model is not associated with obesity, the plasma glucose or triglyceride concentrations never reach levels high enough to be compared with human type 2 diabetes (31), and it does not present insulin resistance, one of the hallmarks of this type of diabetes. The results obtained in STZ-induced diabetic models (1,2) suggested that tungstate restores hepatic glucose metabolism in STZ-diabetic rats (1) and exerts its action at the pancreatic level in nSTZ-diabetic rats (2). After the promising results obtained with STZ and nSTZ rats, we decided to test tungstate treatments on ZDF rats, the genetic type 2 diabetes animal model that most closely resembles the disease in humans (5).

TABLE 2
Triglycerides, cholesterol, and insulin levels of ZLC and ZDF rats

	n	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Insulin (ng/ml)
Healthy Rats				
Untreated	6	165 ± 15	225 ± 16	3.3 ± 0.6
Treated	8	144 ± 14	184 ± 10	3.3 ± 0.7
Diabetic Rats				
Untreated	8	190 ± 21	541 ± 70*	9.3 ± 0.6*
Treated	8	166 ± 16	300 ± 40†	9.9 ± 0.5*

Data are means ± SE. *P < 0.001 compared with untreated and treated healthy animals; †P < 0.001 compared with untreated diabetic animals.

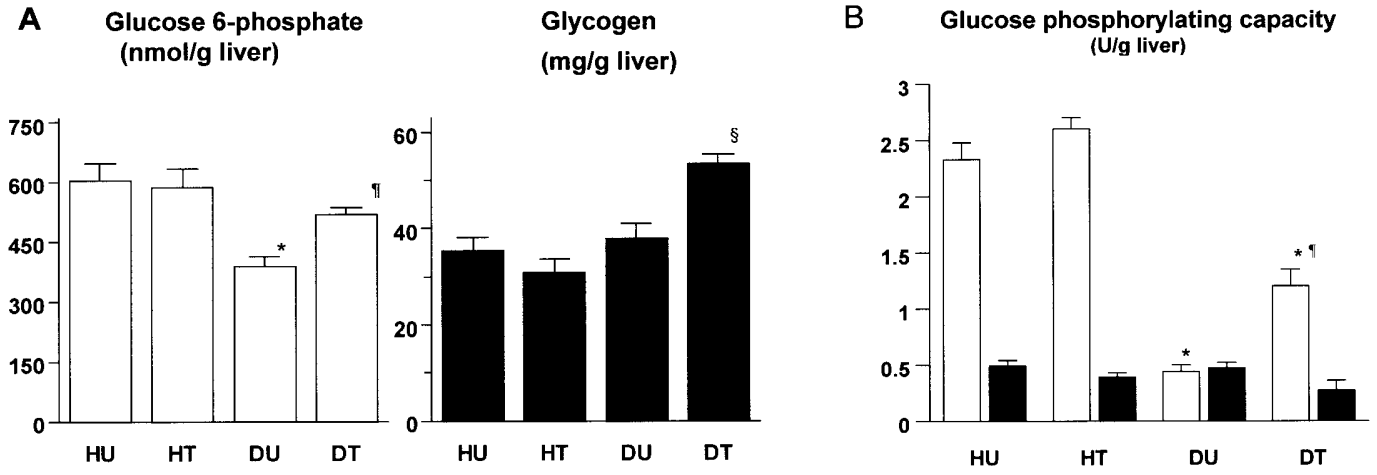


FIG. 4. ***A*: Effects of tungstate treatment on hepatic glycogen and G6P levels in vivo. G6P and glycogen levels were measured in hepatic samples from healthy untreated (HU), healthy treated (HT), diabetic untreated (DU), and diabetic treated (DT) animals. Values are means ± SE (n = 10). *P < 0.01 compared with untreated healthy animals; ¶P < 0.05 compared with untreated diabetic animals. §P < 0.01 compared with untreated healthy and diabetic animals. All of the values were measured at the end of treatment. *B*: In vivo effects of tungstate treatment on hepatic GK (□) and HK (■). GK and HK activities were measured in hepatic samples from healthy untreated, healthy treated, diabetic untreated, and diabetic treated animals. Values are means ± SE (n = 10). *P < 0.0001 compared with untreated healthy animals; ¶P < 0.0001 compared with untreated diabetic animals.**

Our results show that tungstate is effective as an antidiabetic agent in ZDF rats. On one hand, it reduces the high hyperglycemia observed in the untreated animals to values ~200 mg/dl. Although the reduction in glucose levels is not as complete as that in STZ rats, it is clearly a considerable decrease. On the other hand, it retards the appearance of hyperglycemia for about 3 weeks, suggesting that it could be used for the prevention of type 2 diabetes. It is worth noting that the reduction in hyperglycemia with the initiation of tungstate treatment cannot be attributed to weight loss in diabetic animals. Moreover, tungstate does not appear to damage either liver or kidney, as indicated by the reduction in ALT activity, and AST and urea remained unchanged. Most interestingly, tungstate administration causes a dramatic decrease in triglyceride levels in ZDF rats. Thus, tungstate reduces both hyperglycemia and hypertriglyceridemia in these animals. The lowering of triglycerides in plasma undoubtedly contributes to the amelioration of the diabetic state by reducing lipotoxicity. A key point is that tungstate does not induce hypoglycemia in lean animals. This is a most interesting feature of tungstate, and it is clearly a great asset because it does not entail the main disadvantage of insulin treatment: the danger of producing hypoglycemia.

Therefore, after showing the improvement in glucose and lipid homeostasis in ZDF rats treated with tungstate, the metabolic consequences of this treatment at both the hepatic and pancreatic level were further examined. Our results suggest that the mechanism of action of tungstate in these animals is not due to an increase in insulin levels because no detectable changes in their immunoreactive concentrations were observed after treatment. However, because treated rats have similar insulin but lower glucose levels, the pancreas must secrete a similar amount of insulin for a lower glycemia. This observation suggests that tungstate somehow modifies the threshold of insulin secretion in response to glucose.

Liver glucose and lipid metabolism are altered in untreated ZDF rats, and there is a decrease in the activity of some of the principal regulatory enzymes, GK, L-PK, GP*a*, FAS, and a key metabolite, G6P. In contrast, PEPCK is increased. These alterations are normalized by tungstate treatment. Among these defects, the low GK activity probably plays a prominent role (23). Reduced liver GK activity in some obese humans with type 2 diabetes, when compared with nondiabetic normal weight or obese individuals, has been reported (32). Additionally, it has been shown in mice that modest changes

TABLE 3
Effect of tungstate on liver enzymes

		GS			L-PK		
	n	Activity ratio (–G6P/+G6P)	Total specific activity (U/g liver)	GP*a* (U/g liver)	Activity ratio ($V_{0.15}/V$)	Total activity (U/g liver)	FAS (U/g liver)
Healthy Rats							
Untreated	10	0.26 ± 0.05	0.28 ± 0.05	12.8 ± 0.8	0.36 ± 0.02	27.8 ± 3.2	0.47 ± 0.06
Treated	10	0.26 ± 0.04	0.30 ± 0.03	14.2 ± 1.1	0.35 ± 0.02	21.5 ± 2.4	0.63 ± 0.02
Diabetic Rats							
Untreated	10	0.22 ± 0.05	0.27 ± 0.05	9.2 ± 0.8*	0.35 ± 0.03	12.8 ± 1.9*	0.26 ± 0.05*
Treated	10	0.24 ± 0.03	0.29 ± 0.05	14.7 ± 0.8†	0.40 ± 0.04	47.5 ± 3.5†	0.96 ± 0.12†

Data are means ± SE. *P < 0.001 compared with untreated healthy rats; †P < 0.001 compared with untreated diabetic rats.

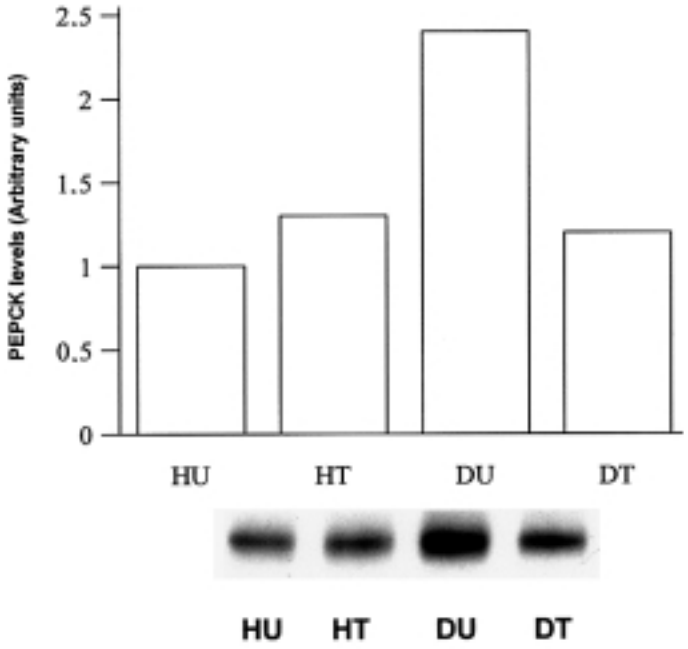


FIG. 5. PEPCK levels after tungstate treatment. PEPCK levels were determined by Western blot analysis of livers of healthy untreated (HU), healthy treated (HT), diabetic untreated (DU), and diabetic treated (DT) rats. Each experimental group consisted of eight animals.

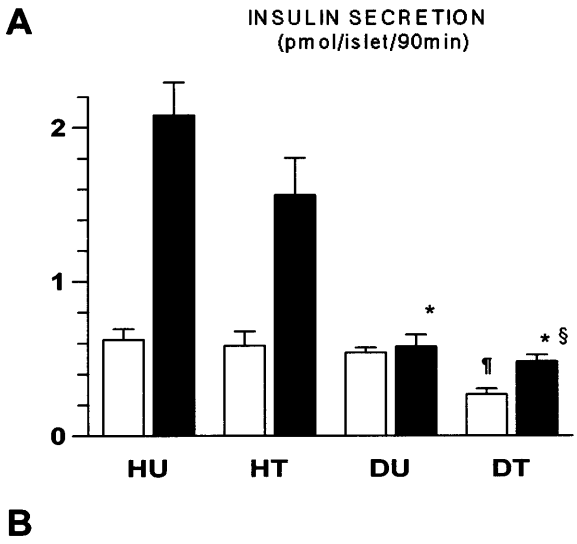
in GK activity cause significant alterations in blood glucose levels (33–38). Furthermore, hepatic GK knock-out mice have moderate hyperglycemia and an impaired insulin secretion in response to glucose (38). Therefore, reduced GK activity is a key feature in the low rate of glucose utilization, which is a characteristic of the diabetic phenotype. The partial restoration of this activity by tungstate probably plays a fundamental role in the mechanism by which this compound reduces glycemia. L-PK is another key enzyme that has an increased activity after tungstate administration to ZDF rats. The restoration in GK and L-PK activities will result in an increase in the glycolytic flux.

Another enzyme that is altered in the hepatic glucose metabolism of ZDF rats is GP*a*, the key enzyme for glycogen degradation. Treatment with tungstate completely restores GP*a* activity to levels similar to those of lean animals. Interestingly, the activity of GS, the regulatory enzyme of glycogen synthesis, was unmodified in ZDF rats compared with controls, and its values did not change in tungstate-treated animals. Hepatic glycogen levels in diabetic animals are similar to those observed in lean controls, but they increase after tungstate administration. The increase in glycogen synthesis may remove part of the excess of circulating glucose present in ZDF rats, thus contributing to reduce glycemia

The augmented PEPCK levels detected in diabetic animals are reverted by tungstate treatment (Fig. 5). This normalization must result in a reduction of gluconeogenesis, which may explain the decreased levels of glucose in fasted tungstate-treated ZDF rats. In conclusion, tungstate treatment also reduces the hepatic potential for glucose output, thus contributing to the lowering of glycemia. Tungstate treatment also restores hepatic FAS activity in diabetic animals, similarly to what has been described for insulin (39–41). However, the plasma triglyceride concentration is decreased by tungstate treatment, unlike the lipogenic capacity (which is increased), and thus suggests a peripheral effect of tungstate on triglyceride removal, possibly through an increased muscular/adipose tissue lipoprotein lipase activity.

The levels of the key metabolite, G6P, are restored in treated diabetic animals probably through the combined effects of the increase in GK activity and inhibition of G6Pase (42). The restoration of G6P is of particular relevance. On one hand, it will contribute to the elevation of glycogen synthesis; on the other hand, it will normalize the mRNA levels of essential enzymes of glucose and lipid metabolism, such as L-PK, acetyl-CoA carboxylase, and FAS, the transcription of which is dependent on the concentration of G6P (43,44).

β-cell function is altered in ZDF untreated rats, with a reduction in the secretion and content of insulin. The small decrease in insulin content and the minute increase in insulin secretion when the islets were stimulated with a shift in glucose concentration after tungstate treatment were



Effect of tungstate on islet performance

	Insulin content (pmol/islet)
Healthy Rats	
Untreated	8.59 ± 0.84
Treated	10.04 ± 0.56
Diabetic Rats	
Untreated	3.68 ± 0.59*
Treated	2.42 ± 0.19¶

FIG. 6. *A*: Glucose stimulus insulin secretion of pancreatic islets from ZDF and ZLC rats. The insulin secretion was measured in islets from healthy untreated (HU), healthy treated (HT), diabetic untreated (DU), and diabetic treated (DT) rats. The experiments were performed at 2.8 mmol/l glucose (□) and at 16.7 mmol/l glucose (■). The values are means ± SE (4 rats and 8 islets with 12 determinations). *P < 0.001 compared with untreated healthy animals; ¶P < 0.01 compared with the remaining group of animals; §P < 0.05 compared with the secretion of 2.8 mmol/l glucose in the treated diabetic animals. *B*: Effect of tungstate on islet performance.

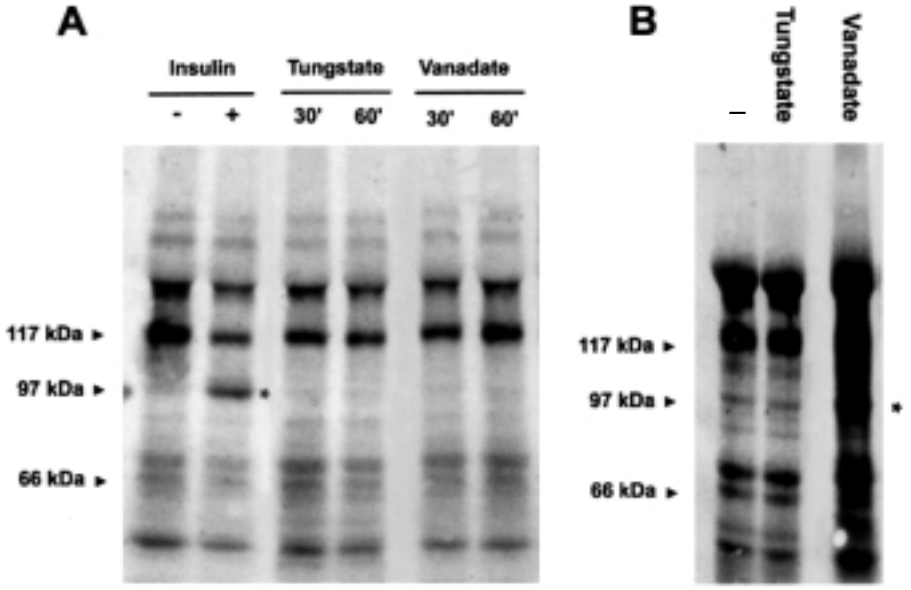


FIG. 7. Protein tyrosine phosphorylation pattern after treatment of primary cultured hepatocytes from ZDF rats with 100 µmol/l sodium tungstate or 50 µmol/l sodium orthovanadate. *A*: Samples of 30 µg total extract from control and treated hepatocytes incubated for 30 and 60 min were immunoblotted with anti-phosphotyrosine antibody. Insulin treatment (100 nmol/l) for 30 min was performed as a positive control. *B*: Samples of 30 µg total extract from control and treated hepatocytes incubated for 10 h were immunoblotted with anti-phosphotyrosine antibody. *Indicates the position of the insulin receptor β subunit.

probably consequences of the decreased glycemia and triglyceridemia and not direct effects of tungstate on islets. The situation of ZDF rats seems to be similar to that of adult STZ rats, in which the hepatic effects of tungstate are prevalent. Interestingly, tungstate exerts (on its own) insulin-like activity on cultured hepatocytes (S. Baqué, unpublished observations).

ZDF rats treated with bis(maltolato)oxovanadium(IV) (BMOV) (8) showed a reduction of hyperglycemia like that which we have described for tungstate. In contrast to our results, BMOV only reduced hypertriglyceridemia in acute treatment in which it was toxic, whereas it did not have any effect on triglyceride levels in chronic treatments. Preliminary results from our group on the molecular mechanisms of tungstate action also show some similarities and dissimilarities with vanadium-derivative treatments. Although they share some aspects from a metabolic perspective, vanadium severely affects other cell functions, such as cell cycle (45; M.C.M., A.B., J.D., J.F.-A., R.G., J.J.G., unpublished observations). In contrast, tungstate appears to have more specific cellular targets. Short-term treatment of primary cultured hepatocytes with tungstate does not seem to directly induce insulin receptor phosphorylation similarly to what has been described in other cell types for vanadium derivatives (46). In striking contrast, incubation with sodium orthovanadate for >3 h, induced generalized tyrosine phosphorylation, including the insulin receptor, whereas the incubation with tungstate did not significantly change the tyrosine phosphorylation profile in these cells. More than likely, tungstate mimics and/or influences insulin effects by directly acting on specific components of the insulin transduction pathway.

In summary, the data obtained in this study show that tungstate is an effective antidiabetic agent in this genetic type 2 diabetes model (ZDF rats), which closely resembles human diabetes.

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