Treatment with sodium tungstate delays diabetes onset and lowers hyperglycemia in the NOD mouse


Introduction: Sodium tungstate is an effective anti-diabetic agent in several animal models of diabetes mellitus in both short- and long-term treatments. Aims: To further characterize its therapeutic application, we studied whether this compound could act in autoimmune diabetes in the NOD (non-obese diabetic) mouse. Material and methods: Four-week-old female mice were given sodium tungstate for 24 weeks. Blood glucose was measured every 2 days throughout the entire experimental period. At the end of treatment, morphometric analysis of the pancreas was performed. Alternatively, diabetic mice were treated with tungstate and liver enzyme activity was determined. Results: We found that tungstate treatment delayed diabetes onset by 6 weeks. In addition, treated mice exhibited lower hyperglycemia at the onset of the disease and this parameter remained low until the end of treatment. Tungstate treatment had no effect on either the severity of insulitis or on β-cell mass. However, tungstate treatment induced a recovery of liver glucokinase and pyruvate kinase activities in diabetic animals. Conclusions: Administration of sodium tungstate to NOD mice corroborates its anti-diabetic properties, delaying diabetes onset and diminishing its incidence. The results indicate that, in the NOD mouse, as in another animal models, the liver is one of the main targets of tungstate actions. Keywords: β cell, insulin-dependent diabetes mellitus, insulitis, liver, pancreas, sodium tungstate.

Abstract

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Introduction

The non-obese diabetic (NOD) mouse develops spontaneous autoimmune diabetes, constituting one of the animal models most similar to human type 1 diabetes mellitus. The autoimmune process begins with an initial phase in which immune cells infiltrate pancreatic ducts and blood vessels, followed by subsequent stages of more aggressive infiltration, with the invasion of the islets of Langerhans (a process called insulitis), leading to their destruction. Increasing interest has been focused on the role played by the β cell in this process. A widely accepted view is that, after autoimmunity, there is a gradual loss of β cells, but cell mass regulatory events are under study. Several studies have focused on the attempt to prevent diabetes in NOD mice by means of prophylactic therapy with insulin, cytokines or anti-inflammatory agents, by modulating β-cell mass or by modifying apoptosis rates. Sodium tungstate has been shown to be a potent anti-diabetic agent in several diabetic rat animal models. When administered orally to streptozotocin-induced diabetic rats, blood glucose levels decreased to normal values, the target tissue being the liver or the pancreas, depending on the animal model studied. However, no studies have involved animal models of genetic autoimmunity. In view of the effectiveness of tungstate in reversing hyperglycemia, we studied whether it could also act in the NOD mouse. Our results demonstrate that tungstate treatment delayed diabetes onset and diminished its incidence. Moreover, treated mice exhibited lower blood glucose levels compared to their untreated counterparts. Parallel to these effects, tungstate-treated mice showed a recovery of some liver enzymes involved in glucose homeostasis.

Materials and methods

Materials

Sodium tungstate (Na₂WO₄) was purchased from Carlo Erba (Milano, Italy); anti-insulin antibody was from ICN Pharmaceuticals (Costa Mesa, CA), and anti-glucagon
and anti-rabbit HRP-conjugated antibodies and the AEC kit were from Sigma (St. Louis, MO). The ApoAlert DNA Fragmentation Assay Kit was purchased from Clontech Laboratories (Palo Alto, CA).

Animals
Four-week-old female NOD/Orl Ico mice (Charles River Laboratories, St. Germain Sur l’Arbresle, France) were used for this study. Animals were randomly separated into two groups: the treated one was given sodium tungstate at 2 mg/ml in drinking water (n= 13), while the other group remained untreated (n= 14). Tungstate treatment lasted 24 weeks. Mice were fed with standard chow (Panlab, Barcelona, Spain) and water ad libitum, and maintained under a 12 h day/night light regime. All mice were housed in accordance with the EC and local government regulations, and procedures were approved by the Research Committee of the Universitat de Barcelona.

Blood glucose, body weight, and food and fluid intake were measured every 2 days throughout the entire experimental period. Mice were monitored for diabetes by measuring blood glucose using a Glucometer Elite (Química-Farmacéutica Bayer S.A, Barcelona, Spain). Animals were considered to be diabetic when the blood glucose values were equal to or above 200 mg/dl.

For the measurement of enzymatic activity, a separate group of diabetic mice was used. Tungstate was administered at 1 mg/ml as usual for 1 week.

Immunocytochemistry
At the end of the treatment, mice were sacrificed and pancreata were carefully removed, fixed in Bouin’s solution and embedded in paraffin. In all studies, 5-µm thickness sections were used. Pancreatic sections were stained for insulin (1:100) or glucagon (1:100), detected with horseradish peroxidase (1:1000) and developed using the AEC chromogen kit. Morphometric analysis was performed using a manual optical picture image analyzer (MOP-01, Olympus, Tokyo, Japan).

Histomorphometry of pancreatic injury
Insulitis was graded using a semiquantitative system, scoring the degree of pancreatic islet infiltration as follows: grade 0 when islets had no signs of infiltration, grade 1 for peri-islet infiltration, grade 2 for intra-islet infiltration and grade 3 for islet destruction. To perform these studies, insulin immunostaining was done and, in the case of grade 3 insulitis (no remaining β cells), glucagon immunostaining was also required.

Detection of apoptosis
Apoptosis was detected on paraffin-embedded pancreatic tissue by the TUNEL method, using the ApoAlert DNA Fragmentation Assay Kit and following the manufacturer’s guide. After performing the apoptosis procedure, samples were co-immunostained with insulin. Images were visualized under a confocal microscope. Fluorescence was monitored with a Leica TCS NT microscope with excitation from the 480 nm line of an argon/krypton filter centered at 530 nm for FITC and above 590 nm for TRITC.

Assays of liver enzymes
Glucokinase and hexokinase activities were measured in fresh liver samples homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with 1 mM EDTA, 100 mM KCl, 300 mM sucrose, and 10 mM β-mercaptoethanol. The homogenates were centrifuged at 10,000 g for 15 minutes at 4 °C. The activities of the supernatants were determined as described. Pyruvate kinase activity was measured in fresh liver samples homogenized in 10 volumes of ice-cold buffer solution at pH 7.4 with 50 mM glycylglycine, 15 mM EDTA, 100 mM KF, and 5 mM potassium phosphate. The homogenates were centrifuged at 10,000 g for 15 minutes at 4 °C, and total pyruvate kinase activity was determined in the supernatants as described.

Statistical analyses
Data were analyzed using SPSS software (version 10.0). Between-group Student’s T or Mann-Whitney tests were carried out. Diabetes incidence was analyzed with the

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<th>Age (weeks)</th>
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<tr>
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Kaplan-Meier test. Results are expressed as mean ± SEM and p values <0.05 were considered significant.

Results
Tungstate treatment delayed diabetes onset and reduced blood glucose

In order to assess the preventive effect of tungstate on the development of diabetes, female NOD mice were treated with sodium tungstate at 4 weeks of age, before any sign of insulitis was evident. The treatment was prolonged until animals were 28 weeks old, a period considered sufficient for the onset of diabetes in all susceptible animals. Tungstate treatment was effective in delaying the onset of diabetes. Thus, while in the untreated group, the first diabetic mice appeared at 12 weeks of age, no mouse in the treated group developed diabetes until the age of 17-weeks-old (figure 1). Regarding diabetes incidence, at 17 weeks of treatment, 28.5% of the animals were diabetic in the untreated group versus only 7.7% in the treated group (table 1). Nevertheless, differences between the two groups in terms of this parameter were not statistically significant at the end of treatment (30.7% vs. 42.8%, treated vs. untreated mice). In addition, the blood glucose level reached at the onset of diabetes was significantly lower in the treated group (255 ± 19 vs. 373 ± 47 mg/dl, treated vs. untreated mice; p<0.05). Moreover, this parameter remained high in the untreated group until the end of the experimental period, whereas mild hyperglycemia was detected throughout in the treated group (282 ± 44 vs. 437 ± 47 mg/dl, treated vs. untreated mice; p<0.05). Nevertheless, no significant differences were observed in plasma insulin levels in treated mice (0.60 ± 0.27 ng/ml) compared to untreated animals (0.47 ± 0.22 ng/ml). Since sodium tungstate has promoted a decrease in food intake in other animal models,11,12 we also measured this parameter in the NOD mice. In agreement with this finding, treated mice had a lower food in-
take (112 ± 8 vs. 149 ± 6 g/kg/day, treated vs. untreated group; p<0.05).

**Tungstate treatment had no effect on β-cell mass**

Histological studies in pancreatic sections were performed at the end of the treatment to elucidate whether the differences observed in treated mice were due to a reduction in the degree of insulitis. No differences were found in islet infiltration in diabetic animals from each group, untreated (2.62 ± 0.32) or treated (2.62 ± 0.22). Interestingly, among the animals that remained normoglycemic in both groups, we detected more mice with pancreatic infiltration in the treated group (80% of the animals) than in the untreated group (43% of the animals). In our study, the immunohistochemical analysis of the pancreas showed no significant difference in either hypertrophy or hyperplasia of the β-cell population (figure 2). As a major event in β-cell destruction in autoimmunity, we then studied whether tungstate modulated apoptosis. Pancreatic sections showed an increase in apoptosis in treated mice, regardless of the metabolic status, compared to untreated mice (figure 3).

**Tungstate treatment recovered liver enzyme activity**

Because the liver is one of the targets of tungstate,11 we studied whether this compound exerted its antidiabetic effect through the recovery of hepatic glucose metabolism. Since some liver enzymes are impaired in diabetic NOD mice,17 we treated them with tungstate. Given that the mice used were not newly diabetic, we administered a lower dose of tungstate (1 mg/ml) for one week. Tungstate treatment significantly increased the activities of glucokinase (0.20 ± 0.13 vs. 0.002 ± 0.002 mU/mg, treated vs. untreated group; p<0.05) and pyruvate kinase (15.61 ± 8.77 vs. 4.57 ± 1.29 mU/mg, treated vs. untreated group; p<0.05), without affecting hexokinase activity (table 2). Concomitantly, in spite of the short treatment period, a reduction in blood glucose levels was observed in treated animals (445 ± 3 vs. 595 ± 5 mg/dl, treated vs. untreated group; p<0.05).

**Discussion**

Administration of tungstate in several rat models of diabetes normalizes hyperglycemia.11-13 Here we demonstrate the effectiveness of tungstate in a mouse model of autoimmune diabetes, the NOD mouse.Remarkably, tungstate treatment produced a delay of up to 6 weeks in
the onset of hyperglycemia. Moreover, although this compound did not completely prevent the development of diabetes, a significant decrease in the incidence of the disease was observed in treated mice, a finding that was more obvious in the middle of the experimental period than at the end. Here we must point out the low incidence of diabetes observed in the animals, explained by the fact that we could not keep mice under specific pathogen-free conditions due to the conditions of our animal facility equipment.

Tungstate-treated mice showed a lower blood glucose level compared to the untreated mice, not only at the onset of diabetes but throughout the treatment period. A reasonable explanation for the lower hyperglycemia observed in the treated animals could be, at least in part, the concomitant decrease (~25%) in food intake (data not shown). However, this effect was examined in rats, where it was observed that tungstate-untreated pair-fed diabetic animals did not diminish hyperglycemia, while diabetic rats treated with tungstate returned to normoglycemia. Thus, it is tungstate per se that directly reduces hyperglycemia. Therefore, we can assume that, in our study, the lowering of hyperglycemia is caused by tungstate administration and not by the parallel decrease in food intake.

To ascertain whether the amelioration of the diabetic status induced by tungstate depended on a mitigation of the autoimmune process, we studied the degree of insulitis. However, the same degree of infiltration was observed in both the treated and untreated diabetic animals. Interestingly, far from reducing insulitis, tungstate increased the number of normoglycemic pre-diabetic mice. One explanation may be that tungstate induced a state of benign autoimmunity, an effect described earlier, that represents a non-destructive response, with the preservation of some functional β cells, thereby enabling the maintenance of normoglycemia. In the normal progression of diabetes in the NOD mouse, the benign autoimmunity becomes malignant, with the destruction of the β cells and the development of insulin-dependent diabetes mellitus (IDDM). Therefore, although they exhibit more infiltration, tungstate-treated mice may have a longer period of benign autoimmunity and, consequently, would remain normoglycemic. In this respect, a study by Palanivel and Sankthisekaran has demonstrated that tungstate has immunomodulatory effects, restoring the number and the functionality of immune cells.

An alternative explanation for the decreased hyperglycemia of the treated mice could reside in the increased β-cell mass, since tungstate treatment has been shown to increase the β-cell population in diabetic rats. In this respect, several authors have emphasized that β-cell mass itself plays an important role in the development of diabetes in the NOD mouse. These changes involve factors that modulate either β-cell replication or apoptosis. In addition, some authors have reported that mice with a remaining β-cell mass above 30% of that of the control mice do not develop diabetes. In our study, even though not statistically significant, tungstate-treated mice displayed a greater β-cell area than the untreated group. On the other hand, Hugues and colleagues postulated that the induction of apoptosis could prevent autoimmune diabetes. In agreement with this hypothesis, in our experiments, we observed a more marked increase in apoptosis in treated animals, which could explain the lower incidence of diabetes.

Similarly to the results obtained in the tungstate-treated STZ rat, we observed an increase in the activity of liver glucokinase and pyruvate kinase, key enzymes in the control of the glycolytic flux. The combination of these two effects results in an increase in glucose disposal and also in a reduction of glucose production, thereby contributing to reduce hyperglycemia.

Little is known about the mechanism involved in tungstate effectiveness as anti-diabetic agent. One of the most widely known properties of tungstate is its activity as a phosphatase inhibitor. These enzymes are involved in numerous cellular events, including apoptosis and metabolism, both of which are affected by the administration of tungstate in NOD mice, as described here. Preliminary data from our laboratory suggest a diminished activity of serine/threonine phosphatases in a β-cell line treated with tungstate (data not shown). Among the proteins dephosphorylated by phosphatases are kinases. Earlier results from our group demonstrated an increase in p38 phosphorylation status in islets from tungstate-treated STZ rats. In this respect, some authors have reported a constitutive phosphorylation of p38 mitogen-activated protein kinase (MAPK), which induces apoptosis in β cells.

Conclusions
Our results demonstrate that tungstate has anti-diabetic effects in autoimmune diabetes in the NOD mouse, de-
laying diabetes onset and reducing its incidence. Moreover, treated mice exhibited lower blood glucose levels compared to their untreated counterparts. The results suggest that, as in other diabetic animal models, tungstate treatment lowers hyperglycemia by stimulating hepatic glucose utilization through increasing hepatic enzyme activity.

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