Modulation of glucose transporters in rat diaphragm by sodium tungstate

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Abstract Oral administration of sodium tungstate is an effective treatment for diabetes in animal models. We examined the effects of 6 weeks of oral administration of tungstate on glucose transporters (GLUT) in streptozotocin-induced diabetic rat diaphragm. Diabetes decreased GLUT4 expression while tungstate treatment normalized not only GLUT4 protein but also GLUT4 mRNA in the diabetic rats. Furthermore, treatment increased GLUT4 protein in plasma and internal membranes, suggesting a stimulation of its translocation to the plasma membrane. Tungstate had no effect on healthy animals. There were no differences in the total amount of GLUT1 transporter in any group. We conclude that the normoglycemic effect of tungstate may be partly due to a normalization of the levels and subcellular localization of GLUT4, which should result in an increase in muscle glucose uptake.

Key words: Diabetes; Glucose transporter; Muscle; Streptozotocin; Rat; Tungstate

1. Introduction

A key role of insulin is to facilitate the uptake of glucose from blood into muscle and adipose tissues [1–4]. In muscle, two glucose transporter isoforms are expressed, GLUT1 and GLUT4. The latter is quantitatively more abundant in adult rat muscle and is distributed among intracellular compartments in the basal state, from where it is rapidly translocated to the plasma membrane in response [3–5] to insulin [2,5,6] or exercise [7,8]. GLUT1 is mainly located at the plasma membrane [4] and is considered to be responsible for basal glucose transport.

Insulin is the cornerstone of treatment for insulin-dependent (type 1) and some forms of non-insulin-dependent (type 2) diabetes mellitus [9]. Sodium tungstate may constitute an alternative to insulin since both long- and short-term treatments with this compound correct the hyperglycemia that occurs in several rat models of diabetes mellitus types 1 and 2 [10–13]. Long-term tungstate treatment also prevents the occurrence of various complications of diabetes in rats, such as morphological changes in kidney and ocular lens, and also reduces mortality but does not cause any undesirable side effects [12]. Tungstate normalizes hepatic carbohydrate metabolism in several animal models [10,11,13] while in neonatally streptozotocin (STZ)-induced diabetic rats it restores β cell function [11]. However, no studies have addressed the effects of this compound in skeletal muscle. Here we studied the effect of tungstate on the glucose transporters GLUT1 and GLUT4 in diaphragm of healthy and STZ-induced diabetic rats. Our results strongly support the notion that the normoglycemic effects of tungstate are partially due to an increase in muscle glucose uptake.

2. Materials and methods

2.1. Materials

Sodium tungstate, STZ and other biochemical reagents were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Animals and experimental design

Adult female Wistar rats, 140–150 g (Granada University breeding colony), were housed at 22°C in a light-controlled (12-h cycle) animal facility and were allowed to eat and drink ad libitum. Experimental diabetes was induced by a single STZ injection (60 mg/kg i.p. in 50 mM sodium citrate, pH 4.5) while control rats received citrate buffer alone. Tail blood glucose levels were measured 10 days after STZ injection and only animals with serum glucose levels greater than 20 mM were used. Treatment began 10 days after STZ injection and was carried out for 6 weeks, during which fluid and food intakes and body weight were measured weekly. Diabetic and healthy rats were divided into two groups. The first (untreated) received drinking water whereas a solution of 2 mg/ml sodium tungstate in water was given to the second group (treated). Serum glycemia was determined by the glucose oxidase–peroxidase method.

At the end of the experiment rats were killed by decapitation between 9.00 and 10.00 h; until this time the animals had free access to food and water. After blood collection, diaphragm was removed, rapidly frozen in liquid nitrogen and kept at −80°C until analysis. Animals were studied in compliance with our institutions' guidelines for animal research and the protocol was approved by the Animal Welfare Committee.

2.3. Subcellular fractionation

Membrane fractions from diaphragm were prepared using a modification of the differential centrifugation method described by Klip et al. [14]. 300 mg of tissue was minced and homogenized (1/10, w/v) by Polytron 2×5 s, setting 4–5, in homogenization buffer (20 mM $NaHCO_3$, 0.25 M sucrose, 5 mM Na_3N, 1 mM leupeptin, 1 mM apoprotinin A, 1 mM pepstatin, pH 7.0) at 4°C. The homogenate was centrifuged at 1200×*g* for 10 min and the pellet was resuspended, homogenized and recentrifuged to remove debris. The combined supernatants were centrifuged at 9000×*g* for 10 min at 4°C for mitochondria and nuclei to sediment. The resulting supernatant was then centrifuged at 190 000×*g* for 60 min at 4°C to obtain the total membrane fraction. The membrane pellets were resuspended in homogenization buffer and the total membrane protein content was measured

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Abbreviations: GLUT, glucose transporter; SSC, sodium chloride/sodium citrate buffer; STZ, streptozotocin

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by the Bradford method [15]. A pool of total membranes corresponding to 6 mg of protein was layered on top of a discontinuous sucrose density gradient (25, 30 and 35% sucrose, w/w) and centrifuged at 150 000×*g* for 16 h at 4°C. Plasma membranes were collected on top of the 25% sucrose layer and low density microsomes on the 35% sucrose layer. Plasma membranes and low density microsomes were washed by 10-fold dilution in 20 mM $NaHCO_3$, 0.25 M sucrose, 5 mM Na_3N and recovered by high speed centrifugation (190 000×*g* for 60 min at 4°C). Membranes were resuspended in homogenization buffer to a final concentration of 1–3 mg protein/ml and stored at −80°C. 5′-Nucleotidase activity was measured as described [16] and cytochrome *c* reductase activity was assayed following Mackler [17] as marker enzymes for plasma membranes and low density microsomes, respectively. Homogenates and membrane fractions were assayed for Ca^{2+}-stimulated ATPase activity to exclude contamination of sarcoplasmic reticulum [18].

2.4. GLUT1 and GLUT4 protein analyses

Electrophoresis in 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels of diaphragm membrane fractions (10 μg for total, 3 μg for plasma membranes and 6 μg protein for low density microsomes) was performed and the resolved proteins were transferred to nitrocellulose filter membranes (Millipore, Bedford, MA, USA). Samples were also analyzed individually by blotting 10 μg protein of each membrane fraction sample onto nitrocellulose membranes using a slot blot apparatus (Bio-Rad, Hercules, CA, USA). After transfer, the membranes were blocked in 5% non-fat milk and incubated with a 1:600 dilution of a polyclonal anti-GLUT1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or a 1:500 dilution of a polyclonal anti-GLUT4 antibody (East Acres Biologicals, Southbridge, MA, USA). The antigen–antibody complexes were detected with horseradish peroxidase-conjugated secondary antibodies using an enhanced chemiluminescence detection system (Amersham, Amersham, UK). In each case, the Western Blot autoradiograms revealed a single band of 45 kDa, compatible with the molecular weight of the transporters. Quantification was performed by densitometry after scanning the autoradiographs with the NIH Image Software [19].

2.5. RNA isolation and Northern blot analysis

Total RNA was isolated from diaphragm of healthy and diabetic rats using a guanidinium thiocyanate method [20]. For Northern blot analysis, 10 μg of RNA was electrophoresed on a 1.0% agarose–5% formaldehyde gel and transferred using a 10× sodium chloride/sodium citrate buffer (SSC) solution to BrightStar-Plus positively charged nylon membranes (Ambion, Austin, TX, USA). For slot blot analysis, 10 μg of the individual RNA samples was spotted onto BrightStar-Plus membranes. After ultraviolet crosslinking, the filters were initially prehybridized for 3 h and then hybridized in Northern Max Prehyb/Hyb buffer (Ambion) for 18 h at 42°C with a random primer labeled cDNA probe for GLUT4 [21]. Finally, the blots were washed once in 2×SSC and 0.1% SDS at room temperature and three times in decreasing concentrations (1× to 0.1×) of SSC and 0.1% SDS at 65°C. Blots were exposed to Kodak Biomax film and intensifying screen at −80°C. The relative amount of mRNA in each sample was quantified by densitometric analysis using the NIH Image Software [19] and the data were normalized to β-actin mRNA [22].

2.6. Statistical methods

Results are expressed as means ± S.E.M. for the indicated number of rats. The data from two independent groups were compared by the Mann–Whitney *U*-test. A P value <0.05 was considered significant.

3. Results

3.1. Effects of tungstate treatment on blood parameters and physical status

After 6 weeks of treatment, glycemia in treated diabetic rats decreased significantly (from 38.3 ± 5.0 to 9.0 ± 0.5 mM), although it remained slightly higher than in their healthy counterparts. Remarkably, tungstate did not modify glucose concentration in healthy rats (6.8 ± 0.3 vs. 6.9 ± 0.4 mM) (Table 1). Untreated diabetic rats increased food and liquid consumption. Tungstate treatment reduced both parameters. As previously reported [12], after 6 weeks of treatment, healthy and diabetic rats showed a decrease in body weight.

3.2. Effects of tungstate treatment on glucose transporters in diaphragm

The amounts of GLUT4 and GLUT1 in total membrane fractions were measured by Western blotting. These assays showed a single band of 45 kDa when probed for each transporter (data not shown). Untreated diabetic rats presented a 40% decrease in the protein content of this transporter when compared to healthy animals (Table 2). Tungstate treatment of diabetic rats completely restored the levels of GLUT4 while it did not alter the levels of this transporter in healthy rats. The amount of GLUT1 protein was not significantly affected by diabetes or tungstate administration (Table 2).

Next, GLUT4 mRNA levels were measured in diaphragm by Northern blot. The assay (Fig. 1) showed a single band of 2.8 kb corresponding to that described for the glucose transporter [21]. Intensity data were normalized to the values of β-actin. A 60% reduction in mRNA levels was observed in the untreated diabetic rats when compared with their healthy counterparts. Treated diabetic rats showed a significantly higher GLUT4 mRNA content than untreated diabetic animals, although it was still slightly lower than in healthy rat samples. However, in healthy rats, treatment did not modify the amount of GLUT4 mRNA.

Table 1
Serum glucose, body weight, fluid, food and tungstate intake in treated or untreated healthy and diabetic rats

	Serum glucose (mM)	Body weight (g)	Food intake (g/kg/day)	Fluid intake (ml/kg/day)	Tungstate intake (mg/kg/day)
Healthy rats					
Untreated (18)	6.8 ± 0.3	222.3 ± 4.6	77.4 ± 3.7	133.6 ± 7.0	
Treated (16)	6.9 ± 0.4	203.2 ± 5.9[e]	89.8 ± 2.2[e]	123.0 ± 3.0	246.1 ± 5.9
Diabetic rats					
Untreated (15)	38.3 ± 5.0[a]	176.0 ± 4.8[a]	138.9 ± 3.1[a]	660.5 ± 16.9[a]	
Treated (12)	9.0 ± 0.5[a,b,d]	151.3 ± 9.0[a,f]	74.0 ± 2.2[b,c]	185.9 ± 12.4[a,b,c]	371.8 ± 24.8[c]

Serum glucose values and body weight are those at the end of the experiment. Food, fluid and tungstate intake are the average consumption of the last week of treatment. Results are expressed as the means ± S.E.M. for the number of rats indicated in parentheses.
[a] $P<0.001$ compared with untreated healthy rats.
[b] $P<0.001$ compared with untreated diabetic rats.
[c] $P<0.001$ compared with treated healthy rats.
[d] $P<0.01$ compared with treated healthy rats.
[e] $P<0.05$ compared with untreated healthy rats.
[f] $P<0.05$ compared with untreated diabetic rats.

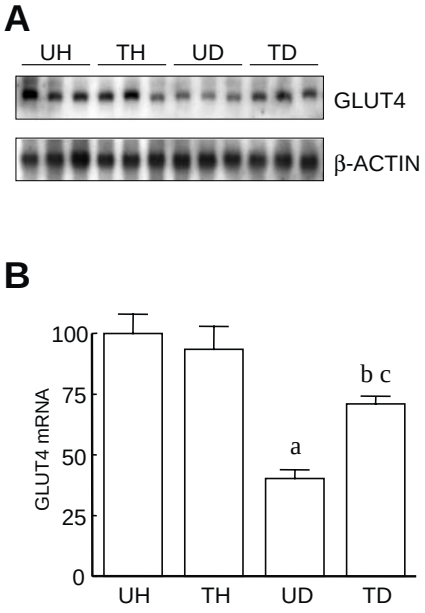

Fig. 1. Effects of sodium tungstate on GLUT4 mRNA levels in rat diaphragm. Diaphragm total RNA was prepared and 10 μg RNA samples were analyzed by Northern blot as described in Section 2. A: Representative Northern blots showing GLUT4 and β-actin mRNAs in untreated healthy (UH), treated healthy (TH), untreated diabetic (UD) and treated diabetic (TD) rats. B: Densitometric quantification of GLUT4 mRNA abundance in rat diaphragm. GLUT4 signal density from untreated healthy animals was assigned a value of 100%. Results were normalized to the β-actin mRNA signal. Values are the means ± S.E.M. ($n = 7$). [a] $P < 0.001$ compared with untreated healthy rats, [b] $P < 0.01$ compared with untreated healthy rats, [c] $P < 0.001$ compared with untreated diabetic rats.

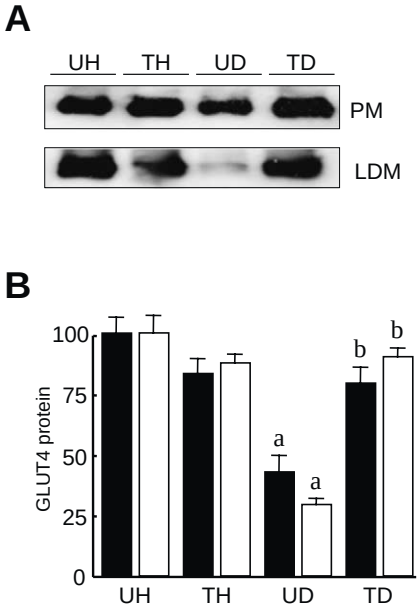

Fig. 2. Effects of sodium tungstate on GLUT4 expression in plasma membranes and low density microsomes from rat diaphragm. Diaphragm subcellular membranes were obtained and protein samples (3 μg of plasma membranes and 6 μg of low density microsomes) were analyzed by SDS–PAGE and Western blotting as described in Section 2. A: Representative Western blots showing GLUT4 protein in plasma membrane (PM) and low density microsomes (LDM) from untreated healthy (UH), treated healthy (TH), untreated diabetic (UD) and treated diabetic (TD) rats. B: Densitometric quantification of plasma (■) and internal membrane (□) GLUT4 protein abundance in rat diaphragm. Signal densities from untreated healthy animals were assigned a value of 100%. Values are the means ± S.E.M. ($n = 5$). [a] $P < 0.001$ compared with untreated healthy rats, [b] $P < 0.001$ compared with untreated diabetic rats, [c] $P < 0.01$ compared with untreated diabetic rats.

3.3. Characteristics of subcellular membrane fractions of rat diaphragm muscle

To assay the distribution of glucose transporters in the plasma membranes and low density microsomes of rat diaphragm, a subcellular fractionation was carried out. The activity of 5′-nucleotidase in the isolated plasma membranes from all rat groups was higher compared with that of the low density microsomes. By contrast, low density microsomes showed a higher cytochrome *c* reductase activity than plasma membranes. In addition, the four groups showed similar marker activities in each of the isolated subcellular fractions, indicating a similar degree of purity regardless of the conditions of each group (Table 3). Ca^{2+}-ATPase, a sarcoplasmic reticulum marker enzyme, was not detectable or barely detectable in the membrane preparations (results not shown), demonstrating absence of significant sarcoplasmic reticulum contamination.

3.4. Effects of tungstate treatment on GLUT4 distribution

To determine whether tungstate modifies the distribution of GLUT4, we quantified it in isolated plasma membranes and low density microsomes. A 60–70% reduction of the protein of this transporter was observed in untreated diabetic rats when compared with healthy counterparts in both fractions. Tungstate treatment induced a significant increase in GLUT4 in diabetic rats, both in plasma membrane and in low density microsomes, reaching levels similar to those of healthy ani-

Table 2
Effects of sodium tungstate on GLUT4 and GLUT1 expression in rat diaphragm

	Healthy rats		Diabetic rats	
	Untreated	Treated	Untreated	Treated
GLUT4 protein	100.0 ± 4.2	90.4 ± 2.3	60.9 ± 2.4[a]	98.4 ± 2.7[b]
GLUT1 protein	100.0 ± 8.7	105.2 ± 5.8	91.5 ± 6.5	91.5 ± 5.9

Effect of sodium tungstate on total membrane fraction GLUT4 and GLUT1 protein in healthy and diabetic rats. Quantification was performed by densitometry after scanning the autoradiographs corresponding to the Western blot determinations, as described in Section 2. Signal densities from untreated healthy animals were assigned a value of 100% for each transporter. Results are expressed as the means ± S.E.M. for eight rats per condition.
[a] $P < 0.001$ compared with untreated healthy rats.
[b] $P < 0.001$ compared with untreated diabetic rats.

Table 3
Characterization of membranes isolated from diaphragm of untreated and treated healthy and diabetic rats

	5′-Nucleotidase (nmol/min/mg protein)		Cytochrome *c* reductase (nmol/min/mg protein)	
	Plasma membranes	Low density microsomes	Plasma membranes	Low density microsomes
Healthy rats				
Untreated	595.7 ± 65.5	158.3 ± 24.8	26.9 ± 6.4	223.0 ± 15.04
Treated	570.1 ± 36.7	167.5 ± 26.9	28.5 ± 6.0	191.5 ± 18.0
Diabetic rats				
Untreated	655.8 ± 71.9	190.5 ± 9.9	36.5 ± 7.9	243.6 ± 62.5
Treated	663.2 ± 92.5	177.7 ± 21.4	27.5 ± 8.6	162.2 ± 18.1

Plasma membranes and low density microsomes were isolated from rat diaphragms as described in Section 2. The isolated fractions were assayed for protein content, 5′-nucleotidase and cytochrome *c* reductase activities. Results are for five independent preparations from each group. Each assay was carried out in triplicate. Results are expressed as the means ± S.E.M.

mals. Tungstate had no effect on GLUT4 protein in either of these fractions of healthy rats (Fig. 2).

4. Discussion

Tungstate is an effective agent for normalizing glycemia in STZ-diabetic rats [10,12]. Here we studied the effect of this compound on the glucose transporters GLUT1 and GLUT4 in rat diaphragm. We show that tungstate produces a normalization of the levels of GLUT4, indicating that an increase in glucose transport in muscle is also involved in the mechanism by which tungstate exerts its antidiabetic action. GLUT1, which was not altered by diabetes, was unaffected by tungstate administration.

GLUT4 protein and GLUT4 mRNA content decreased in untreated diabetic rats. GLUT4 expression is down-regulated when there is a relative insulin deficiency, such as in STZ-induced diabetes and chronic fasting [24]. Our results demonstrate that tungstate treatment to diabetic rats partially restores GLUT4 mRNA levels, which results in the complete normalization of the protein levels. Similar results have been shown in adipocytes isolated from STZ-induced diabetic rats treated with vanadate [23,25]. The differences in the degree of restoration of protein and mRNA levels could be explained by the fact that this gene is regulated extensively at the post-transcriptional level. In several situations, including dietetic treatment of diabetes [26], the GLUT4 protein and/or mRNA can be stabilized, thereby increasing the total amount of the transporter. Tungstate treatment may induce a similar process.

The effects of tungstate on GLUT4 distribution in skeletal muscle of STZ-diabetic rats clearly differ from those of other antidiabetic agents. Our results show that oral administration of sodium tungstate increases the amount of this transporter in both low density microsomes and plasma membranes. Vanadium derivatives increase the GLUT4 content in low density microsomes but not in plasma membranes of adipose tissue [27,28] or skeletal muscle [29]. Troglitazone, a therapeutic agent used to treat type 2 diabetic patients with insulin resistance, increases the GLUT4 content of plasma membrane by inducing its translocation from intracellular pools [30], without causing a net increase in the total amount of the transporter.

Insulin increases glucose uptake in its target tissues, muscle and fat, by inducing the translocation of GLUT4 from an intracellular pool to the plasma membrane [3–5]. Our results show that tungstate also triggers the translocation of GLUT4 in treated diabetic rats since in plasma membranes this compound induced a significant increase in GLUT4 content, which reached levels that were similar to those in untreated healthy rats. Tungstate had no effect on the protein expression of this transporter in internal or plasma membranes of healthy rats.

In conclusion, tungstate, like insulin, exerts pleiotropic effects that also involve the muscle. The increase in GLUT4, which should result in augmented muscle glucose uptake, may also contribute to the normoglycemic effect of this compound.

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