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Effect of vanadate on glucose transporter (GLUT4) intrinsic activity in skeletal muscle plasma membrane giant vesicles

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Abstract

Maximally effective concentrations of vanadate (a phosphotyrosine phosphatase inhibitor) increase glucose transport in muscle less than maximal insulin stimulation. This might be due to vanadate-induced decreased intrinsic activity of GLUT4 accompanying GLUT4 translocation. Thus, the effect of vanadate ($NaVO_3$) on glucose transporter (GLUT4) intrinsic activity (V_{max} = intrinsic activity × [GLUT4 protein]) was studied in muscle plasma membrane giant vesicles. Giant vesicles (average diameter 7.6 μm) were produced by collagenase treatment of rat skeletal muscle. The vesicles were incubated for 1.5 h with concentrations of vanadate ranging from 3 to 40 mmol l^{-1} at 34°C before being used for determination of glucose transport. The dose–response curve showed that vanadate decreased the specific D-glucose uptake by a maximum of 70% compared with a control preparation. The vanadate-induced decrease in glucose uptake was not due to a decrease in number of vesicles. To further verify the apparent vanadate-induced decrease in GLUT4 intrinsic activity, the kinetics of glucose transport were also examined. In the presence of 10 mmol l^{-1} vanadate the V_{max} and K_m were decreased ($P < 0.05$, $n = 6$) 55% and 60%, respectively, compared with control. The plasma membrane GLUT4 protein content was not changed in response to vanadate. It is concluded that vanadate decreased glucose transport per GLUT4 (intrinsic activity). This finding suggests that regulation of glucose transport in skeletal muscle can involve changes in GLUT4 intrinsic activity.

Keywords: Glucose transport; GLUT4 intrinsic activity; Skeletal muscle; Vanadate

1. Introduction

Vanadate (a phosphotyrosine phosphatase inhibitor) fully mimics insulin-stimulated glucose transport and GLUT4 protein translocation in adipocytes [1–3]. However, the vanadate-induced increase in glucose transport is lower than a maximal insulin stimulation in skeletal muscle [4–6]. This might be explained by vanadate decreasing the GLUT4 intrinsic activity by inhibiting a phosphotyrosine phosphatase. Furthermore, a substantial number of studies [7–19] on glucose transport in adipocytes also suggest that a change in the GLUT4 intrinsic activity by phosphorylation is a prerequisite for maximal insulin-stimulated glucose uptake. Moreover, it has been shown in skeletal muscle that an increase in cAMP by epinephrine administration reduced the glucose uptake by approx. 50% despite an increase of GLUT4 proteins in the plasma membrane [20]. Dibutyryl cAMP reduced the GLUT4-mediated glucose uptake by 50% when GLUT4 was expressed in Chinese hamster ovary cells with unaltered concentrations of GLUT4 proteins in the plasma membrane [21]. All taken together, these findings suggest that changes in GLUT4 intrinsic activity could be mediated by changes in GLUT4 protein phosphorylation status.

Intrinsic activity (k_{cat}: catalytic turnover number) is defined by $V_{max} = k_{cat} \times$ [GLUT4 protein] [22]. Thus, measurements of GLUT4 intrinsic activity in intact skeletal muscle require a fixed concentration of GLUT4 proteins in the plasma membrane. We used a recently described method [23] for producing skeletal muscle plasma membrane giant vesicles, which enables precise determination of vanadate-induced changes in GLUT4 intrinsic activity without changes in the GLUT4 protein concentration.

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2. Materials and methods

Nycodenz was from Nycomed AS, Norway. Percoll® was from Pharmacia, LKB Sweden. Phloretin, PMSF and collagenase (type VII) were from Sigma, St. Louis, USA. Aprotinin (Trasylol) was from Bayer, Leverkusen, Germany. D-[^{3}H]Glucose and L-[^{14}C]glucose were from New England Nuclear, Boston, MA. All other chemicals were of analytical grade.

Preparation of plasma membrane vesicles. Plasmalemma vesicles were produced by a modification of the procedure by Burton et al. [24], as described previously [23]. In brief, male Wistar rats (240–260 g), fed ad libitum, were anesthetized with sodium pentobarbital (5 mg per 100 g body weight) and the gastrocnemius-soleus-plantaris muscles, together with quadriceps femoris muscles, from both hindlimbs were removed, and cut longitudinally into thin slices (1–2 mm) and washed briefly in KCl-Hepes buffer (140 mmol l^{-1} KCl, 5 mmol l^{-1} Hepes, pH 7.8) with 0.37 mg ml^{-1} PMSF (phenylmethylsulfonyl fluoride) added. Subsequently, the muscle slices were incubated for 40 min at 34°C in a final volume of 20 ml of KCl-Hepes buffer containing 0.041 mg ml^{-1} highly purified collagenase together with the proteinase inhibitors aprotinin (0.01 mg ml^{-1}) and PMSF (0.37 mg ml^{-1}). During incubation spontaneous formation of vesicles ranging in diameter from 3–40 μm occurs. To purify and concentrate the vesicles, Percoll was added to the suspension of vesicles and cell debris to a final concentration of 16%. The suspension was placed at the bottom of six centrifugation tubes and overlayered by 3 ml 4% nycodenz (w/v) in KCl-Hepes buffer and by 1 ml of KCl-Hepes buffer. The tubes were spun at $40 \times g$ for 45 min at room temperature, whereupon the vesicles were harvested between the two top layers, diluted in KCl-Hepes buffer and pelleted at $830 \times g$ for 30 min. For subsequent protein determination the vesicles were stored at −20°C. Protein concentrations were determined in duplicate by using the bicinchoninic assay [25].

Microscopy. Diluted aliquots of membrane vesicles were placed in a blood cell counting chamber. The vesicles were allowed to settle for 15 min and then examined with a Zeiss phase-contrast microscope.

Measurements of glucose transport in membrane vesicles. Glucose transport (influx) was measured at room temperature and at zero-trans conditions as described in [23]. In brief, 30 μl of membrane vesicles were placed in a 1.5 ml Eppendorf tube and mixed with 10 μl of a solution containing D-[^{3}H]glucose, L-[^{14}C]glucose and various concentrations of unlabelled D-glucose and mannitol in KCl-Hepes buffer. Time-course of 5 mmol l^{-1} zero-trans D-glucose uptake is linear for the first 30 s [23]. Thus, 30 s (initial rate of D-glucose uptake) was used when the D-glucose concentration was varied between 2–40 mmol l^{-1} final concentration. The mannitol concentration was varied reciprocally so that the total concentration of sugars was 40 mmol l^{-1}. After the designated time, entry of glucose was stopped by addition of 1.0 ml ice cold stop solution (200 μmol l^{-1} phloretin in KCl-Hepes buffer) and the mixture was spun for 1 min in a high speed centrifuge ($17\,000 \times g$). The supernatant was aspirated and the bottom part of the Eppendorf tube, containing the pellet, was cut off and counted with a Packard Tri Carb 2000CA liquid scintillation counter. Specific D-glucose uptake was calculated after subtracting the distribution space for L-glucose from the D-glucose space in the pellet [23]. Estimation of maximum velocity (V_{max}) and the constant K_m in the Michaelis–Menten equation was done by curve fitting using the least-squares method.

Incubation with vanadate. Because the plasma membrane proteins in the vesicles are oriented 'right side out' [24,26], it was important that vanadate gained access to the intravesicular volume. Thus, the vesicles were incubated for 1.5 h with different concentrations of $NaVO_3$ (ranging from 3 to 40 mmol l^{-1}) at 34°C before the vesicles were spun down (30 min) and subsequently used for glucose transport experiments. It should be noted that vanadate is poorly permeable so the actual intravesicular concentration can be significantly lower. The control preparation was incubated at 34°C with 20 mmol l^{-1} mannitol in order to avoid a osmotic-induced difference in glucose transport. In the dose–response experiment, the vesicles were incubated at 34°C with 3–40 mmol l^{-1} of $NaVO_3$ and 0–80 mmol l^{-1} of mannitol so that the total osmotic pressure was constant. The glucose uptake (after 1 min) was not different after an incubation with 80 mmol l^{-1} mannitol compared with a control preparation incubated in mannitol-free buffer (data not shown). The vesicles were sedimented at $830 \times g$ for 30 min, and the pellet was resuspended in the respective vanadate- or mannitol-buffer before the vesicles were used for measurement of glucose transport.

Western blot. The plasma membrane vesicles were incubated for 2 h at 34°C in the presence and absence of 10 mM vanadate. The plasma membrane vesicles were washed once in a hypertonic buffer (300 mmol l^{-1} KCl, 25 mmol l^{-1} pyrophosphate, 10 mmol l^{-1} Hepes, pH 7.4) followed by a wash in a hypotonic buffer (10 mmol l^{-1} Tris, pH 7.4) and pelleted (90 min, 50000 RPM, Beckman 70.1 Ti-rotor) as described by Ploug et al. [23]. Approximately 15% of the vesicle protein was recovered as membrane protein with no significant difference in membrane protein recovery in response to the vanadate treatment. The membrane proteins were separated using SDS-PAGE, electrotransferred to an Immobilon P membrane (Milipore), immunoblotted, and GLUT4 antibody-antigen complexes were visualised within the linear range by the enhanced chemoluminiscense (ECL) detection kit (Amersham) as previously described by Ploug et al. [23]. The GLUT4 antibody was a mouse monoclonal antibody produced against a synthetic peptide corresponding to the 13 C-terminal amino acids of GLUT4 [23].

Statistics. The statistical tests used were the unpaired

t-test, and the ANOVA test. Significance was set at the 0.05 level of confidence. Results are presented as means ± standard error of the mean (S.E.).

3. Results

The dose–response curve showed that vanadate inhibited the specific D-glucose uptake measured as total D-[^{3}H]glucose uptake subtracted the unspecific L-[^{14}C]glucose. Vanadate decreased the glucose uptake by maximally 70% compared with the control preparation (Fig. 1). The dose–response curve displayed saturation and the maximal inhibition of glucose uptake was observed at 10 mmol l^{-1}. Furthermore, by visual inspection in a phase-contrast microscope it was verified, that 2 h of incubation with 40 mmol l^{-1} vanadate at 34°C did not affect the number or the appearance of the vesicles compared with a control preparation.

To further verify that vanadate inhibits glucose transport, vesicles were incubated for 2 h with 10 mmol l^{-1} vanadate at 34°C before being used for time-course studies of glucose uptake. The time-course of 5 mmol l^{-1} D-glucose zero-trans entry into membrane vesicles in the absence or presence of vanadate is shown in Fig. 2. In agreement with the data in Fig. 1, vanadate caused a marked inhibition of D-glucose uptake in the vesicles.

The glucose concentration dependence of the initial rate of glucose transport (measured as 30 s uptake) is shown in Fig. 3. In the control situation the V_{max} was 358 ± 50 pmol mg^{-1} protein s^{-1} and the K_m was 24 ± 5 mmol l^{-1}. In the presence of 10 mmol l^{-1} vanadate, the V_{max} was significantly reduced to 162 ± 18 pmol mg^{-1} protein s^{-1} and the K_m was significantly decreased to 10 ± 1 mmol

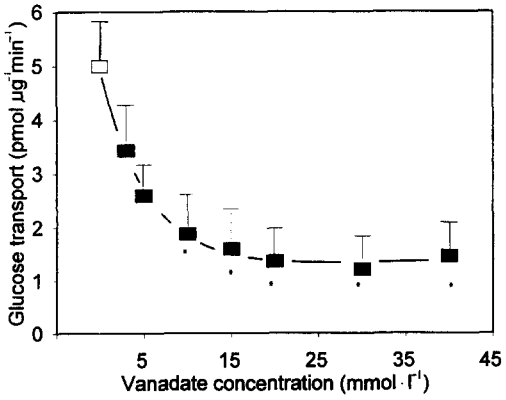

Fig. 1. Dose–response curve for vanadate action on glucose uptake. Vesicles were incubated at 34°C for 2 h with 3–40 mmol l^{-1} of $NaVO_3$. D-Glucose uptake was measured after 1 min of incubation with 5 mmol l^{-1} D-glucose, 0.5 µCi D-[^{3}H]glucose, and 0.1 µCi L-[^{14}C]glucose. Values are means + S.E. of five determinations, each determination in triplicate. □, Control preparation; ■, vanadate preparation. * $P < 0.05$ compared with control.

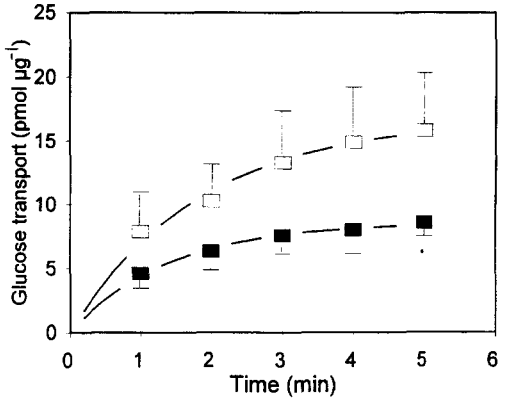

Fig. 2. Time-course of specific D-glucose uptake measured at 5 mmol l^{-1} D-glucose, 0.5 µCi D-[^{3}H]glucose, and 0.1 µCi L-[^{14}C]glucose. Values are means ± S.E. of three determinations, each in duplicate. □, Control preparation; ■, vanadate preparation. * $P < 0.05$ compared with control.

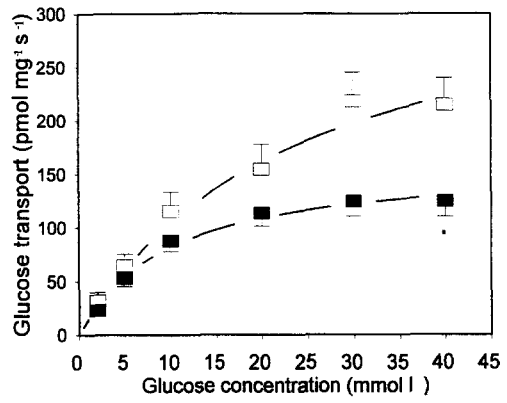

Fig. 3. Initial rate of specific D-glucose uptake as a function of external D-glucose concentration. The vesicles were incubated 2 h with 10 mmol l^{-1} of $NaVO_3$. Glucose uptake was determined in duplicate at 2, 5, 10, 20, 30 and 40 mmol l^{-1} of D-glucose. In the control the K_m was 25 ± 5.1 mmol l^{-1} and V_{max} was 358 ± 50 pmol mg^{-1} total vesicle protein s^{-1} and with vanadate the K_m was 10 ± 1.1 mmol l^{-1} and V_{max} was 162 ± 18 pmol mg^{-1} total vesicle protein s^{-1}. Values are means ± S.E. of six preparations. □, Control preparation; ■, vanadate preparation. The lines were drawn by using the calculated values of V_{max} and K_m.

l^{-1} (mean ± S.E., $n = 6$, * $P < 0.05$ for both V_{max} and K_m).

The GLUT4 protein content in the vesicles was measured by Western blot after 2 h of incubation in the presence and absence of 10 $mmol^{-1}$ of vanadate. Vanadate did not induce any significant change in the GLUT4 content (control 4.41 ± 0.25 arbitrary units; vanadate 4.49 ± 0.27 arbitrary units, mean ± S.E., $P = 0.71$, $n = 8$).

4. Discussion

In this study of GLUT4 intrinsic activity, we have utilized a recently developed and characterized muscle plasma membrane vesicle preparation [23]. Characteriza-

tion of the vesicles showed that the vesicle membrane components are almost entirely of plasma membrane origin since the plasma membrane marker K^+ activated *p*-phenylphosphatase was enriched 37-fold [23]. Furthermore, the vesicles are devoid of contamination with sarcoplasmic reticulum, T-tubuli or mitochondrial membranes [23,26] and GLUT1 could not be detected [23]. We found that the plasma membrane GLUT4 protein content was not changed in response to vanadate. Therefore the possibility of GLUT4 protein translocation can be, as expected, excluded in this system. Hence, the preparation is well suited for studying changes in glucose transport via alterations in GLUT4 intrinsic activity.

Vanadate has a wide range of metabolic effects [27]. Vanadate partially mimics the effects of insulin by stimulating GLUT4 translocation and glucose transport in rat adipocytes [1–3]. In particular, vanadate is a well-known phosphotyrosyl protein phosphatase inhibitor and insulin mediates at least some of its effects via an insulin-receptor associated tyrosine kinase. However, vanadate probably mimics the actions of insulin at a post-receptor level [1].

Recent reports suggest that dephosphorylation of the GLUT4 isoform promotes its translocation from the intracellular storage site to the plasma membrane in adipocytes [15,18]. In contrast, increased phosphorylation of the GLUT4 is reported to decrease GLUT4 intrinsic activity in adipocytes [7,9–12,14,15]. In particular, it was found that streptozotocin-induced diabetes decreased the insulin-stimulated glucose transport by an increased GLUT4 protein phosphorylation [15]. The increased GLUT4 phosphorylation was restored by insulin therapy, whereas vanadate was ineffective [15]. In skeletal muscle, both insulin and vanadate stimulate glucose transport and yet vanadate is less effective in stimulating glucose transport compared with a maximal insulin stimulation [4–6]. Interestingly, Henriksen et al. [4] also found that insulin and vanadate in combination increased glucose transport to a similar extent as insulin alone in skeletal muscle. The finding by Henriksen et al. [4] may suggest that the GLUT4 protein is refractory to a vanadate-induced increase in phosphorylation status when insulin is present. This is in accordance with an insulin-induced decrease in GLUT4 protein phosphorylation as found in adipocytes [7,9–12,14,15]. All previous findings [4–7,9–12,14,15] taken together with the present findings, suggest that vanadate could inhibit a phosphotyrosyl phosphatase, thereby increasing the GLUT4 phosphorylation status and decreasing the GLUT4 intrinsic activity.

In contrast to our study, Okumura et al. [28] reported that vanadate enhanced glucose transport 4–5-fold in rat sarcolemmal vesicles. One explanation for this discrepancy could be differences in the method for producing plasma membrane vesicles. The preparation method of Okumura et al. [28] employs initial homogenization of the muscle. Vigorous homogenization produces contamination of the plasma membrane with tissue of non-muscular origin, such as nerves and blood vessels. Since GLUT1 is localized in tissues with barrier functions [29], a substantial part of GLUT1 originates from perineural sheaths in muscle [30]. Thus, the preparation of Okumura et al. [28] probably contains GLUT1. This is supported by the fact that the preparation of Okumura et al. [28] is in fact prepared using the method of Grimditch et al. [31], which method is reported to give a preparation that does contain GLUT1 [32,33]. In contrast, our preparation method does not include homogenization of the tissue. Instead, vesicles are produced by bleb formation of the plasma membrane [23]. Therefore, our preparation contains very little if any GLUT1 [23]. Since Okumura et al. [28] reported that vanadate enhanced glucose uptake 5–6-fold in erythrocyte ghosts, which only contain GLUT1 [34], it is very likely that vanadate increased the glucose transport in their preparation by increased GLUT1 intrinsic activity.

Due to the formation of plasma membrane blebs in our preparation method, substantial amounts of cytosolic proteins are enclosed — and trapped inside the plasma membrane spheres. In fact, approximately 90% of the total protein is lost after destruction of the vesicular structure, allowing removal of soluble intravesicular proteins [23]. Thus, our vesicle preparation could contain different proteins (including phosphotyrosine phosphatases) involved in increasing the GLUT4 intrinsic activity.

In conclusion, in a giant vesicle preparation derived from rat sarcolemma, vanadate decreased glucose transport by reducing the GLUT4 intrinsic activity.

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