Marked depletion of GLUT4 glucose transporters in transverse tubules of skeletal muscle from streptozotocin-induced diabetic rats

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Abstract The principal goal of the present study was to determine the subcellular content of GLUT4 in diabetic rat muscle, and to test the hypothesis that a reduced abundance of the transporter protein in transverse tubules is responsible for impaired glucose utilization in that tissue. GLUT4 protein levels were measured in hindlimb muscle homogenates as well as in subcellular membrane fractions enriched with either plasma membranes, transverse tubules, or GLUT4-containing intracellular membranes from control and diabetic (streptozotocin-induced) rats. GLUT4 protein contents in diabetic muscle homogenates was reduced by 30% as compared to control rats. Subcellular fractionation experiments revealed that GLUT4 contents in transverse tubules-enriched fractions was markedly decreased (by 55–60%) in skeletal muscle of diabetic animals whereas no significant reductions in GLUT4 abundance was observed in the plasma membrane fraction. Moreover, GLUT4 was markedly depleted (by 45%) in the GLUT4-enriched intracellular membrane fraction. These results indicate that GLUT4 is markedly depleted in both the intracellular pool and in the cell surface membranes in muscle of STZ-diabetic rats. Most strikingly, this study demonstrates that transverse tubules and not the plasma membrane are the main sites of cell surface GLUT4 depletion in diabetic muscle.

Key words: Diabetes; Skeletal muscle; GLUT4; Transverse tubule; Plasma membrane

1. Introduction

Glucose transport accross the cell surface is believed to be the rate-limiting step for muscle glucose disposal both in normal and diabetic individuals [1,2]. In skeletal muscle, the transport of glucose is predominantly mediated by the GLUT4 glucose transporter. It is well documented that insulin acutely stimulates glucose transport by inducing the translocation of GLUT4 transporters from tubulo-vesicular intracellular structures to the plasma membrane [3–6]. However, more recent studies have shown that the GLUT4 glucose transporter is also localized to transverse tubules in rat skeletal muscle [6–9]. The transverse tubules are deep invaginations of the plasma membrane that are involved in excitation–contraction coupling in skeletal muscle. Moreover, we have previously reported that insulin induces the translocation of GLUT4 to both the transverse tubules and the plasma membrane in rat skeletal muscle [9]. These studies, combined with the fact that transverse tubules constitute the largest (>60%) area of the cell surface in muscle cells [10,11], led us to propose that these tubular structures significantly contribute to the entry of glucose into myofibers, both in the basal and stimulated conditions. This hypothesis was recently confirmed by Dudek et al. [12] who found that insulin increases cell surface photolabelled GLUT4 transporters to the transverse tubules using an autoradiographic method.

Skeletal muscle glucose utilization is decreased in both insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) (see [13] for a review). A defective expression or regulation of GLUT4 translocation is thought to be responsible for the defect in muscle glucose disposal. It has been reported that GLUT4 protein and mRNA levels are decreased in skeletal muscle of several rodent models of both genetically and experimentally induced diabetes [14–17]. In contrast, several studies have shown that GLUT4 expression (GLUT4 mRNA and protein) is not reduced in skeletal muscle of IDDM and NIDDM individuals as compared to normal subjects [18–22] with the exception of one study with severely obese type II diabetic individuals [23]. However, it may be possible that GLUT4 protein abundance in human muscle may be specifically decreased in the cell surface membranes, where glucose transport takes place. Indeed, it has been reported that the reduction in GLUT4 content is more pronounced in isolated plasma membranes than in total membranes in diabetic rat models of IDDM and NIDDM [17,24]. Recently, two groups have specifically looked at this possibility in human muscle from NIDDM subjects. Whereas Vogt et al. [25] found a 40–50% decrease in GLUT4 in their plasma membrane fraction, Lund et al. [26] reported no differences in plasma membrane GLUT4 content between muscles of control and diabetic individuals. However, the possibility that GLUT4 is depleted in muscle transverse tubules of diabetic animals or IDDM and NIDDM subjects remains to be investigated.

The principal goal of the present study was to determine whether GLUT4 content is reduced in isolated transverse tubules from diabetic skeletal muscle, using the streptozotocin (STZ)-treated rat as a model of diabetes. To do so, we have used a novel membrane fractionation procedure that allows the simultaneous isolation of plasma membranes and transverse tubules in distinct subcellular fractions from the same muscle sample.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Montreal) weighing ~200 g were injected i.p. with 65 mg/kg of STZ (Sigma) freshly dissolved in 20 mM citrate buffer (pH 4.5). The control group was injected with citrate buffer alone. Tail vein glucose levels were monitored on a daily basis with a glucometer (One touch basic, Lifescan) and only rats presenting glycemia ≥12 mM were considered diabetic and used in the following experiments. Seven days after STZ injection, diabetic and

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control rats were fasted for 6 h, sacrificed and hindlimb muscles (soleus, plantaris, tibialis, gastrocnemius and quadriceps) were rapidly excised, cleaned of extraneous tissues and frozen in liquid nitrogen. The muscles were kept at −80°C until use for membrane preparation. Whole blood was kept to determine serum glucose and insulin levels using a glucose analyser (Beckman, model 2300 StatPlus) and by RIA using a commercial kit (Incstar, Stillwater, MN), respectively.

2.2. Subcellular membrane fractionation

Plasma membranes (PM), transverse tubules (TT1 and TT2) and intracellular membranes (L-IM) were isolated by subcellular fractionation using a procedure recently developed in our laboratory [27] and partly based on previous methods [4,5,9]. This new protocol allows the simultaneous isolation of plasma membranes and transverse tubules in distinct fractions from the same original muscle sample. Furthermore, as shown in the present study, the procedure yields a novel intracellular membrane fraction markedly enriched with GLUT4 but devoid of markers for plasma membranes or transverse tubules (see Section 3).

Hindlimb muscles (~9 g) were minced in buffer A (10 mM $NaHCO_3$, pH 7.0, 0.25 M sucrose, 5 mM NaN_3, 100 μM phenylmethylsulfonylfluoride). At this point, a sample was taken and homogenized at high speed and was used as the 'homogenate' fraction. The minced muscles were homogenized twice using a Polytron equipped with a PT20 probe and a Kinematica speed control (Brinkman Instruments) at a precise low setting of 5.0 for 5 s. The resulting homogenate was centrifuged at $1300 \times g$ for 10 min. The $1300 \times g$ supernatant was centrifuged at $9000 \times g$ for 10 min and then at $190,000 \times g$ for 1 h. The $190,000 \times g$ pellet was resuspended in buffer A and applied on discontinuous sucrose gradients (25, 32 and 35%, w/w) and centrifuged at $150,000 \times g$ for 16 h. Membranes at the sample/25% [plasma membranes (PM)] and 32/35% [transverse tubules (TT1)] interphases were recovered, diluted with sucrose-free buffer A and centrifuged at $190,000 \times g$ for 1 h.

The pellet from the above $1300 \times g$ spin was resuspended in buffer B (0.5 M LiBr, 50 mM tris, pH 8.5) and stirred for 4 h. The LiBr-treated membranes were centrifuged first at $1200 \times g$ for 5 min and then at $10,000 \times g$ for 30 min and again at $30,000 \times g$ for 1 h. The pellet was resuspended in buffer C (0.15 M KCl, 5 mM $MgSO_4$, 20 mM HEPES, pH 6.8), applied on 10, 27 and 35% discontinuous sucrose gradients (w/w) and centrifuged at $150,000 \times g$ for 16 h. Membranes at the 27/35% interphase [(transverse tubules (TT2)] were recovered. The supernatant of the $30,000 \times g$ spin was used to isolate the intracellular membrane fraction (L-IM) as follows. It was centrifuged at $190,000 \times g$ for 1 h and the resulting pellet was resuspended in buffer A. The resuspended membranes were applied on a 10/40% discontinuous sucrose gradients and centrifuged at $150,000 \times g$ for 16 h. Membranes at the 10/40% interphase [(intracellular membranes (L-IM)] were collected from the gradients, diluted with sucrose-free buffer A and were centrifuged at $190,000 \times g$ for 1 h. All fractions were resuspended in buffer A and used fresh for enzymatic activity measurements or kept at −80°C until used for Western blot analysis. 5′-Nucleotidase activity in the isolated membrane fractions was measured as previously described [28].

2.3. Western blot analysis and antibodies

Membranes (10 μg of proteins unless otherwise indicated) were subjected to SDS-PAGE on a 7.5% polyacrylamide gel as described by Laemmli [29], and Western blotting was performed as previously described [5].

Polyclonal antibodies against the GLUT1 and GLUT4 glucose transporters were purchased from East Acres Biologicals (Southbridge, MA). The monoclonal antibody 1IF7 generated against the alpha1-DHPr was kindly supplied by Dr. K. Campbell (University of Iowa). The monoclonal antibody against the α1-Na/K-ATPase was a kind gift from Dr. K. Sweadner (Massachusetts General Hospital).

3. Results

Rats injected with streptozotocin were clearly diabetic after 7 days, as evidenced by the presence of fasting hyperglycemia (1.9-fold over control) and hypoinsulinemia as compared to citrate-injected animals (Table 1). As expected, diabetic animals gained less weight than controls and consequently, hindlimb muscle weights were slightly reduced after one week of diabetes

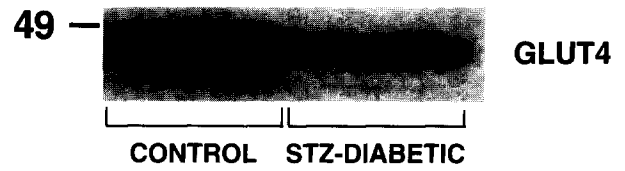

Fig. 1. GLUT4 protein contents in muscle homogenates from control and STZ-diabetic rats. The figure shows GLUT4 protein levels in muscles of 5 control and 5 diabetic rats. A molecular weight standard is shown on the left. Homogenates of mixed hindlimb muscles were prepared and Western blot analysis was performed using 30 μg of proteins. Following an overnight incubation at 4°C with primary antibodies, immunoblots were incubated with either anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Amersham). Immunoreactive bands were detected by the enhanced chemiluminescence method. Autoradiographs were quantified by laser scanning densitometry using the 'BioImage-Visage 110S' software from Millipore (Ann Arbor).

(Table 1). However, this had no effect on the membrane yields of the various fractions, as indicated in Table 2.

The effect of STZ-diabetes on GLUT4 protein contents in skeletal muscle homogenates is shown in Fig. 1. GLUT4 was detected as a single band migrating at 45–46 kDa in both control and diabetic rat samples. Muscle total GLUT4 content was reduced (by 30%) in muscle of diabetic rats as compared to controls (rel. densit. units control vs. diabetic samples: 126 ± 7 vs. 88 ± 6, $n = 7$, $P < 0.01$). In contrast, the relative amounts of DHPr, a transverse tubule-specific protein, were not different between control and diabetic rat homogenates (data not shown).

We next examined GLUT4 membrane distribution and contents in isolated membrane fractions. Membrane fractions were first characterized using 5′-nucleotidase activity (Table 2) and the immunologic detection of markers of both the plasma membrane (α1-Na/K-ATPase and GLUT1) and the transverse tubules (DHPr) (Fig. 2, Table 2). Neither the diabetic state nor the minor differences in muscle weight significantly affected 5′-nucleotidase activity in these membrane fractions (Table 2). As expected, the enzyme activity was mostly abundant in the PM fraction, confirming that this fraction is enriched with plasma membrane vesicles. The enzyme was also detected in TT1 and TT2 fractions but the activities were much lower and not very different than that measured in homogenates (~10 pmol/mg/min). Furthermore, 5′-nucleotidase was not detectable in the LiBr-released intracellular membrane fraction (L-IM), at least when using up to 40 μg of proteins in the assay. However, in preliminary experiments using larger amounts of muscle from control rats, we could detect some activity using 60–70 μg of L-IM membrane proteins. This activity was lower

Table 1
Physiological parameters of control and STZ-diabetic rats

	Control	STZ-diabetic
Initial body weight (g)	205 ± 1	208 ± 3
Final body weight (g)	275 ± 2	221 ± 7**
Body weight gain (g)	70 ± 2	13 ± 7**
Muscle weight (g)	9.83 ± 0.75	7.60 ± 0.48*
Fasting glycemia (mM)	8.44 ± 0.13	15.87 ± 3.86*
Fasting insulinemia (nM)	0.95 ± 0.24	0.25 ± 0.06**

Data are mean ± S.E. of seven rats in each group.
*$P < 0.05$, **$P < 0.01$ as analysed by Student's t-test for unpaired comparisons.

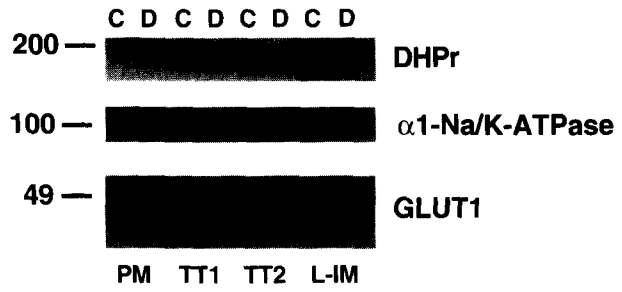

Fig. 2. Subcellular distribution of plasma membrane and transverse tubule markers in isolated membrane fractions and effects of STZ-diabetes. Fractions enriched with either plasma membranes, transverse tubules, or intracellular membranes were isolated from muscles of control (C) and diabetic (D) rats as described in Section 2. The relative contents of DHPr, α1-Na/K-ATPase and GLUT1 proteins were assessed by Western blotting using specific antibodies (see legend of Fig. 1). Mean values from seven individual experiments are shown in Table 2. Molecular weight standards are shown on the left.

or similar to homogenates confirming that plasma membranes were not enriched in that fraction (data not shown). We have also verified that LiBr treatment did not interfere with the enzyme assay by incubating membranes with or without 0.5 M LiBr (buffer B) for 4 h, followed by assessment of 5′-nucleotidase activity. No differences were observed between LiBr-treated and untreated membrane samples (data not shown).

The membrane distribution and levels of the α1-subunit of the Na/K-ATPase, a plasma membrane marker [5], and of the DHPr, a transverse tubule marker [9], are shown in Fig. 2. The α1-Na/K-ATPase was mainly present in the PM fraction, in agreement with the enrichment in 5′-nucleotidase activity in that fraction. Lower, but detectable levels of the subunit could also be detected in TT1 but not in TT2 or L-IM fractions. In sharp contrast, the DHPr was mainly detected in TT1 and TT2 fractions and could be barely detected in the PM or the L-IM fraction. The latter fraction was devoid of markers of either plasma membrane or transverse tubules, thus confirming that vesicles of intracellular origin were isolated in that fraction. Moreover, the GLUT1 glucose transporter was only detected in the plasma membrane fraction (Fig. 2), confirming previous immunoblotting and immunocytochemical studies [5,30]. Importantly, STZ-diabetes had no significant effects on any of these proteins (see mean values in Table 2), indicating that diabetes did not lead to non-specific changes in the recovery of either plasma membranes or transverse tubules in the above fractions.

A representative immunoblot showing GLUT4 protein amounts in surface membranes and intracellular membranes isolated from muscle of STZ-diabetic and control rats is depicted in Fig. 3A. The mean values from seven individual experiments are presented in Fig. 3B. GLUT4 was markedly enriched in the intracellular membrane fraction (L-IM), confirming that it represents a GLUT4-enriched intracellular pool. When compared to the plasma membrane fraction, GLUT4 abundance was slightly higher in TT1 transverse tubule fraction and significantly greater in the TT2 fraction in both control and diabetic muscles. Remarkably, GLUT4 protein levels were markedly decreased in transverse tubule fractions (by 55–60%) of diabetic rats as compared to non-diabetic controls (Fig. 3A,B). The GLUT4-enriched intracellular fraction was also markedly depleted (by 45%) of GLUT4 in the diabetic state. On the other hand, GLUT4 abundance in the plasma membrane fraction was slightly but not significantly reduced. In average, we observed a small 20% reduction in GLUT4 content in that fraction. The effect of diabetes on GLUT4 protein levels was maintained when data were expressed per total fraction since membrane yields were not affected by the diabetic state (Table 2) (data not shown).

4. Discussion

It has been shown that basal glucose transport is decreased in perfused hindlimb muscles of STZ-diabetic rats [24]. It has also been reported that GLUT4 is decreased in the plasma membrane of STZ-diabetic rats [24,31]. However, when both hindlimb glucose uptake and GLUT4 abundance in the plasma membrane were compared in the same animals, the reduction in plasma membrane GLUT4 content was found to be very small (−18%) as compared to the impairment in basal glucose uptake (−70%) in the hindlimbs [24]. In the present study, we have also observed a slight, albeit not significant, 20% reduction in plasma membrane GLUT4 abundance.

More importantly, GLUT4 protein levels in transverse tubules, the main area of the muscle cell surface envelope, has never been examined in skeletal muscle of diabetic rats. The results of the present study demonstrate for the first time that transverse tubules are markedly depleted of GLUT4 transporters in skeletal muscle of STZ-diabetic rats. GLUT4 was decreased by > 50% in both fractions enriched with transverse

Table 2
Enzymatic and immunologic characterization of isolated membranes and effects of STZ-diabetes

Fractions		Yield (μg/g muscle)	5′ AMP (nmol/mg/min)	α1-Na/K (rel. units)	DHPr (rel. units)	GLUT1 (rel. units)
PM	CTL	28 ± 1	165.8 ± 22.9	1606 ± 428	73 ± 26	135 ± 15
	STZ	30 ± 4	175.0 ± 11.4	1806 ± 354	88 ± 17	129 ± 28
TT1	CTL	197 ± 11	40.6 ± 4.3	280 ± 83	879 ± 183	ND
	STZ	267 ± 30	38.9 ± 6.9	327 ± 69	825 ± 185	ND
TT2	CTL	77 ± 26	15.5 ± 5.8	ND	1044 ± 399	ND
	STZ	92 ± 25	15.7 ± 5.6	ND	884 ± 224	ND
L-IM	CTL	35 ± 11	ND	ND	305 ± 57	ND
	STZ	35 ± 12	ND	ND	268 ± 46	ND

Data are mean ± S.E. of seven membrane preparations in each experimental group.
PM, plasma membranes; TT, transverse tubules; L-IM, LiBr-released intracellular membranes; ND, non-detectable.
No significant differences were observed between control and STZ-diabetic animals.

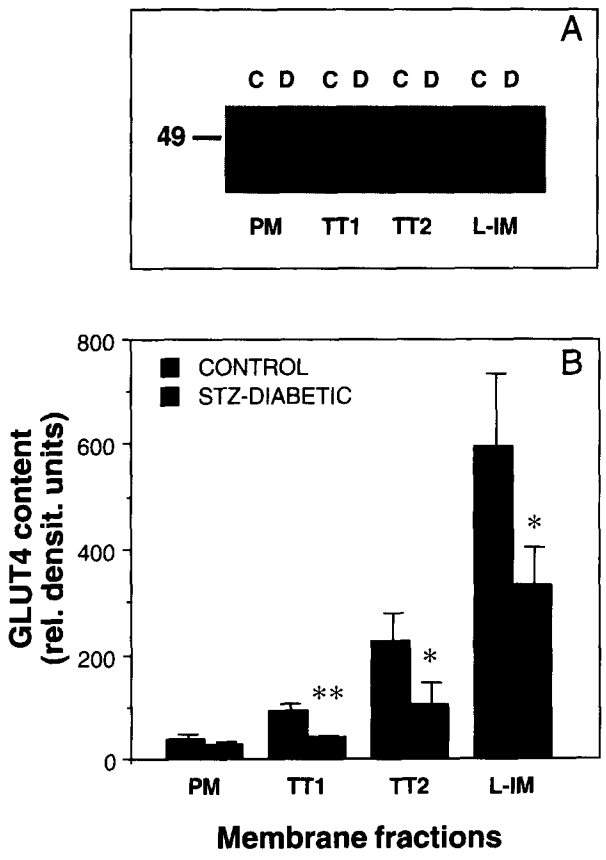

Fig. 3. GLUT4 protein levels in plasma membrane (PM), transverse tubules (TT1 and TT2) and intracellular membrane (L-IM) fractions isolated from muscles of control (C) and diabetic (D) rats. (A) Representative immunoblot, (B) mean ± S.E. of seven individual membrane preparations, each performed with one control and one diabetic rat. Scanning data are expressed relative to GLUT4 levels in crude muscle membrane samples that were run on parallel on every gels. $^{*}P < 0.05$, $^{**}P < 0.01$ as analysed by Student's t test for unpaired comparisons.

tubules whereas the transporter abundance was not significantly reduced in the plasma membrane fraction (Fig. 3). On the other hand, GLUT1 is not present in the transverse tubules and its content in the plasma membranes is not changed in STZ-diabetic rats. Thus, our data suggest that the defective basal glucose uptake in muscle of STZ-diabetic animals is mainly explained by a decreased number of GLUT4 operating in the transverse tubules. However, direct experimental evidence will only come with measurements of glucose transport fluxes in isolated transverse tubules vesicles.

In agreement with previous studies [24,31], GLUT4 was also found to be significantly depleted from the intracellular membrane fraction in STZ-diabetic rats. This fraction is markedly enriched with GLUT4 but devoid of markers of both plasma membranes and transverse tubules, even when compared to muscle homogenates. We have recently shown that this intracellular fraction represents an insulin-responsive intracellular pool from which GLUT4 is translocated to both the plasma membrane and the transverse tubules in non-diabetic rats [27]. Whether the translocation of GLUT4 from this GLUT4-depleted intracellular fraction to either plasma membranes or transverse tubules is impaired in the STZ-diabetic rat model remains to be investigated.

The differences in GLUT4 levels between the two TT fractions (Fig. 3) despite similar levels of DHPr (Fig. 2, Table 2) is intriguing. This difference was seen both in normal and diabetic animals. Transverse tubule membranes recovered in fraction TT1 could be easily dissociated from the muscle bulk by mild homogenization whereas TT2 contains transverse tubule vesicles that were released using LiBr. Another difference is that the α1-subunit of the Na/K-ATPase could be detected in TT1 but not TT2 fractions. It has been previously demonstrated that transverse tubules are not always joined to the SR in mammalian skeletal muscle [32]. It is therefore possible that TT1 membranes are non-junctional and, thus, derived from tubules not joined to the cisternal SR, thereby explaining their release by mild homogenization. On the other hand, TT2 may contain junctional transverse tubules that can only be released by high-salt treatment. The non-junctional tubules would not be involved in the excitation–contraction coupling but still contribute to increase the availability, transport and utilization of extracellular substrates such as glucose deep into the muscle cells.

In summary, the results of the present study show that GLUT4 protein content is markedly depleted in both the intracellular pool and in the cell surface membranes in muscle of STZ-diabetic rats. Furthermore, this study provides the first experimental evidence that cell surface GLUT4 depletion is much greater in the transverse tubules than in the plasma membrane in skeletal muscle of STZ-diabetic rats.

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