Direct evidence of fiber type-dependent GLUT-4 expression in human skeletal muscle

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Gaster, M., P. Poulsen, A. Handberg, H. D. Schrøder, and H. Beck-Nielsen. Direct evidence of fiber type-dependent GLUT-4 expression in human skeletal muscle. Am J Physiol Endocrinol Metab 278: E910–E916, 2000.—GLUT-4 expression in individual fibers of human skeletal muscles in younger and older adults was studied. Furthermore, the dependency of insulin-stimulated glucose uptake on fiber type distribution was investigated. Fiber type distribution was determined in cryosections of muscle biopsies from 8 younger (29 yr) and 8 older (64 yr) healthy subjects, and estimates of GLUT-4 expression in individual fibers were obtained by combining immunohistochemistry and stereology. GLUT-4 was more abundantly expressed in slow compared with fast muscle fibers in both younger (P < 0.007) and older (P < 0.001) subjects. A 25% reduction of GLUT-4 density in fast fibers (P < 0.001) and an unchanged GLUT-4 density in slow fibers were demonstrated in older compared with younger subjects. Insulin-stimulated glucose uptake rates measured by hyperinsulinemic, euglycemic clamp were not correlated with the fraction of slow fibers in the young (r = -0.45, P > 0.25) or in the elderly (r = 0.11, P > 0.75) subjects. In conclusion, in human skeletal muscle, GLUT-4 expression is fiber type dependent and decreases with age, particularly in fast muscle fibers.

GLUCOSE TRANSPORT across the cell membrane in skeletal muscle is mediated by the glucose transporter proteins GLUT-1 and GLUT-4 (3). The GLUT-1 glucose transporter isofrom is believed to support basal glucose transport (35, 42), whereas the GLUT-4 isoform increases glucose transport in response to insulin and contraction. Insulin and contractions induce translocation of GLUT-4 from intracellular storage vesicles to the plasma membrane and to the transverse tubules (20, 33, 34).

In rodents, it is well established that the glucose uptake capacity is greater in red oxidative muscles than in white glycolytic muscles (13, 17, 29, 34). One underlying mechanism appears to be a higher level of GLUT-4 expression, both intracellularly (13, 17) and at the plasma membrane (34). In human skeletal muscle, glucose uptake after submaximal insulin stimulation is positively correlated with the percentage of type I fibers and negatively correlated with the percentage of type IIb fibers (31). In accordance with the results from rodents, this has been attributed to a higher expression of GLUT-4 in red oxidative fibers than in glycolytic fibers (34). Zierath et al. (47) showed, in a study of in vitro incubated human muscle strips from healthy subjects, that the insulin-stimulated increase in glucose uptake over basal is strongly correlated, both positively with the percentage of type I muscle fibers and negatively with the percentage of type IIa fibers. However, inconclusive results regarding the relationship between fiber type distribution and GLUT-4 content in human muscle biopsies have been reported (2, 24, 25). Andersen et al. (2) found no correlation between fiber type and GLUT-4 content, whereas Houmard and co-workers (24, 25) described a weak relation between fiber type composition and GLUT-4 content. Thus, in human skeletal muscle, it has not yet been established whether GLUT-4 expression varies with fiber type or whether a fiber type-dependent GLUT-4 expression might contribute to interindividual variations of sensitivity and/or responsiveness of glucose uptake. The finding of a fiber type dependency of GLUT-4 expression in rodents arose from immunoblotting studies of muscles selectively enriched with a single fiber type (36). Human skeletal muscles consist of mixtures of all the fiber types (27), and thus immunoblotting studies are unlikely to reveal fiber type-dependent expression of GLUT-4.

Our aim was to develop a method that allows studies of GLUT-4 expression in individual type-identified human muscle fibers. The relationship between GLUT-4 expression and fiber type in human skeletal muscles in younger and older adults was studied using the combined method of immunohistochemistry and stereology. Furthermore, the dependency of insulin-stimulated glucose uptake on fiber type distribution was elaborated.

METHODS

Study subjects. Sixteen healthy volunteers (Table 1) participated in the study. The protocol was approved by the local ethical committees of Funen and Vejle counties, and informed consent was received from all subjects before participation.

Oral glucose tolerance test. Subjects underwent a standardized 75-g oral glucose tolerance test (OGTT) after a 10-12-h overnight fast. Peripheral venous blood samples were drawn...
before and 30 and 120 min after glucose ingestion. An exclusion criterion was a 120-min glucose value higher than 11 mmol/l.

Euglycemic hyperinsulinemic clamp. The participants underwent a hyperinsulinemic euglycemic clamp (23). The experiments started after a 10-h overnight fasting. The 2-h (0 to +120 min) clamp period was initiated by a 9-min squared priming infusion, followed by a constant infusion (40 mU·m²·min⁻¹) of insulin (Actrapid; Novo-Nordisk, Bagsvaerd, Denmark). Plasma glucose concentration was maintained constant at euglycemia by glucose infusion (180 g/l). Plasma glucose concentration was monitored in arterialized blood every 5–10 min with an automated glucose oxidase method (Glucose Analyzer 2, Beckman Instruments, Fullerton, CA). The basal steady state was from −30 to 0 min before the onset of insulin infusion, and the insulin-stimulated steady-state period was predefined as +90 to +120 min during clamps. Whole body glucose utilization was determined from the variable glucose infusion during the steady-state period of the clamp. Glucose uptake rates were expressed per kilogram of fat-free mass determined by dual-energy X-ray absorptiometry scanning (39).

Muscle biopsy. Biopsies from the vastus lateralis muscle were obtained by the method of Bergström (4) during the basal and the insulin-stimulated steady-state periods of the euglycemic hyperinsulinemic clamp. Muscle tissue was mounted in Tissue Tek OCT-compound (Sakura, Torrance, CA) and frozen. The orientation of the specimens was random.

Morphometry. All procedures were performed on 5-µm unfixed cryosections of human skeletal muscle biopsies. Primary antibodies were rabbit anti-GLUT-4 antibody, AB1346 (Chemicon, Temecula, CA); mouse anti-skeletal fast myosin antibody, clone MY32 (Sigma, St. Louis, MO) reacting with type 2 fibers as determined by the ATPase reaction; and mouse anti-skeletal slow antibody, clone WB-MHCs (Novocastra, Newcastle, UK). Immunoblotting of single antigens was performed using the horseradish peroxidase (HRP)-labeled streptavidin biotin technique (40). Immunoblotting of GLUT-4 and either slow myosin or fast myosin (double immunoblotting) was performed using a modified, simultaneous "indirect-indirect" immunoenzymatic technique (46). Briefly, dry cryosections were incubated for 1 h with a mixture of rabbit anti-GLUT-4 antibody (1:1,600) and mouse anti-skeletal fast myosin antibody (1:64,000) or mouse anti-skeletal slow myosin antibody (1:50). After a rinsing with buffer, sections were incubated for 30 min with a mixture of alkaline phosphatase (AP)-conjugated goat anti-mouse antibody, D486 (1:20) (DAKO, Glostrup, Denmark) and biotinylated goat anti-rabbit antibody, E432 (1:200) (DAKO). Sections were washed and treated for 30 min with HRP-conjugated streptavidin P397 (1:300) (DAKO). Histochemical development of enzyme activities was performed sequentially. First, AP activity was visualized using New Fuchsin as a chromogen (5). After 30 min of incubation, development was terminated by thorough rinsing. Subsequently, HRP activity was detected using nickel (Ni)-enhanced diaminobenzidine (DAB) as a chromogen (43). Incubation time for DAB-Ni was 10 min. Finally, sections were counterstained with ethylene green, dehydrated, and mounted with pertex (Histolab, Vastra Frölunda, Sweden). All dilutions of antibodies were done in 1% BSA (Sigma), and Tris-HCl-buffered saline, pH 7.4, was used for rinsing. In control sections, the primary antibodies were omitted or replaced by primary mouse IgG isotype antibodies (Coulter, Miami, FL).

Morphometry. An estimate of the expression of GLUT-4 in slow and fast muscle fibers was obtained by stereological analysis of immunostained sections. The material used for quantification consisted of two double immunohistochemically stained sections from each biopsy, one stained for fast myosin and GLUT-4 and one for slow myosin and GLUT-4. All sections used for quantification were stained simultaneously to ensure uniform technical procedures. The sections were analyzed in a CAST GRID system (Olympus, Denmark). In each section more than 20 fields, size 0.1134 mm², including >200 muscle fibers, were automatically randomly sampled for analysis (14). In each field the number of fibers was counted, and their diameters were determined as the lesser muscle diameter (9). The fraction of slow and fast fibers was calculated as the number of either slow or fast fibers divided by the total number of counted fibers. GLUT-4 was quantified as the number of cell surface-related grains resulting from the GLUT-4 immunocytochemistry. Only clearly separated grains were counted individually, conglomerates being counted as one grain. The counts of GLUT-4 in slow fibers were performed in the sections stained for GLUT-4 and fast myosin, and vice versa, to avoid interference of the myosin immunostaining for fiber type identification with the GLUT-4 counts. The GLUT-4 counts were correlated to the areas occupied by the slow and fast fibers, estimated in the same fields by use of a grid with 48 equidistant points. The GLUT-4 density in each fiber type could be expressed as the number of grains per point, each point corresponding to 0.1134 mm²/48. The volume fraction of each fiber type was determined as the number of points associated with a fiber type divided by the total number of points counted. Mean GLUT-4 concentration in a muscle was estimated as the sum of each fiber volume fractions multiplied by their corresponding mean GLUT-4 density, and in the description that follows it is designated as "estimated GLUT-4 concentration."

Western blotting. Of each muscle biopsy, 10–30 mg were homogenized in 50 mM Hepes, pH 7.6, and 250 mM sucrose buffer containing 20 mM molybdate, 1.5 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 1 µM pepstatin A, 1 µM leupeptin, and 400 millikrein-inhibitor units of Trasylol/ml for 2 × 10 s. A crude membrane fraction was isolated from the supernatant as described in Ref. 6, and its protein concentration was determined by the Bradford method (Bio-Rad, Copenhagen, DK). During membrane preparation, a pellet from an insulin-stimulated biopsy from an elderly subject was lost and, because of a lack of muscle, could not be replaced. Five micrograms of protein from each of the crude membrane fraction were separated by SDS-PAGE and blotted to nitrocellulose membranes. GLUT-4 immunoreactivity was visualized by chemiluminescence using RalRGT (Charles River, Southbridge, MA) as primary antibody and was quantified by densitometric scanning.

Statistical analysis of data. Data in text, tables, and figures are given as means ± SE. Statistical analyses were performed with INSTAT 2.01 (GraphPad). Nonparametric statis-
tical analyses of data were used: Mann-Whitney test for unpaired comparisons and Spearman’s rank correlation coefficient \( r \) for analysis of covariance. \( P < 0.05 \) was considered significant.

Statistical analyses of method. Measurements of GLUT-4 density in muscle fibers by combined immunohistochemistry and stereology, as well as estimates of GLUT-4 concentration in muscle biopsies, were analyzed with respect to their sensitivity, precision, and accuracy. The smallest detectable difference among estimates of GLUT-4 concentration is 0.33, and among GLUT-4 densities it is 0.31 when the significance level is 0.05, and a risk of type 2 error (overlooking differences) of 0.10 is accepted. Thus the sensitivity of our method is high, allowing differences of 10–15% to be detected. The coefficients of variation (CV) of the estimates of GLUT-4 concentration are 9.3% in the elderly and 4.3% in the younger subjects, and the CVs of the determinations of GLUT-4 concentration are 9.3% in the elderly and 4.3% in the younger subjects, respectively. One available estimate of the accuracy of our method is the identical and expressed no dependency of type or age (68.8 \( \pm \) 2.6 \( \mu \)m, young vs. elderly, slow fibers, and 70.8 \( \pm \) 3.9 vs. 65.6 \( \pm \) 3.2 \( \mu \)m, young vs. elderly, fast fibers) (P > 0.9) (Table 2). Because the diameters of slow and fast fibers are similar, and because the fiber area fraction determined by point counting is directly equivalent to the fiber volume fraction, the observed fiber-related differences in GLUT-4 immunoreactivity found when correlated to cross-sectional area can be extended to include a fiber type-related difference when correlated to fiber volume, circumference, and surface area.

GLUT-4 and insulin. Insulin stimulation by the hyperinsulinemic clamp caused no significant changes in GLUT-4 densities (P > 0.11) in any of the fiber types (Table 2). Furthermore, no significant correlation between estimated GLUT-4 concentration and insulin-stimulated glucose uptake rate was found, either for the young (r = −0.52, P = 0.20) or for the older (r = 0.5, P = 0.22) subjects (data not shown).

GLUT-4 and age. The mean of GLUT-4 density in the basal and the insulin-stimulated states was used for studies of the impact of age on GLUT-4 expression. A reduction of 25% of GLUT-4 density in the fast fibers (2.12 \( \pm \) 0.15 vs. 2.85 \( \pm \) 0.17 nos./point, P < 0.001) and an unchanged GLUT-4 density in slow fibers (3.10 \( \pm \) 0.11 vs. 3.25 \( \pm \) 0.07 nos./point, P = 0.14) were demonstrated in the older subjects compared with the young (Fig. 2). Mean GLUT-4 density in the younger females was 3.28 \( \pm \) 0.15 compared with 3.17 \( \pm \) 0.06 in males, in slow fibers, and 2.93 \( \pm \) 0.06 vs. 2.77 \( \pm \) 0.04 in fast fibers. In the elderly subjects, slow fiber GLUT-4 density was 3.25 \( \pm \) 0.1 vs. 2.64 \( \pm \) 0.15, and fast fiber GLUT-4 density was 2.14 \( \pm \) 0.05 vs. 2.03 \( \pm \) 0.07, females vs. males (Fig. 2). These observations, of less GLUT-4 in male subjects of either age group and for both fiber types, could not be verified by statistical analyses, because of the limited number of study participants. The estimated GLUT-4 concentrations were significantly higher in muscle from younger compared with elderly subjects (3.02 \( \pm \) 0.05 vs. 2.56 \( \pm \) 0.08, P < 0.003) (Fig. 3A).

GLUT-4 by Western blotting. Insulin stimulation in vivo caused no changes in GLUT-4 expression (Table 3) in muscle biopsies from either the young (2.06 \( \pm \) 0.63 vs. 2.11 \( \pm \) 0.38, optical density (OD)/mg tissue, P > 0.9) or the older subjects (1.37 \( \pm \) 0.25 vs. 1.36 \( \pm \) 0.21, OD/mg tissue, P > 0.9). Thus the means of GLUT-4 expression in the basal and insulin-stimulated states were used for studies of the impact of age on GLUT-4 expression (Fig. 3B). The GLUT-4 content in muscle biopsies from older subjects was not significantly reduced compared with the younger subjects (1.36 \( \pm \) 0.16
vs. 2.08 ± 0.36, OD/mg tissue, P = 0.12). To address further the significance of the increased GLUT-4 density in slow fibers, analyses of covariation between the GLUT-4 content in crude membranes and the fraction of slow fibers in cryosections of the same biopsies were performed. No significant correlation could be demonstrated either in the younger (r = 0.089, P = 0.75) or the elderly (r = 0.21, P = 0.45) subjects. Furthermore, insulin-stimulated glucose uptake rate and GLUT-4 protein per milligram tissue were not correlated in the younger (r = 0.32, P = 0.22) or the older subjects (r = 0.068, P = 0.8).

Comparison of GLUT-4 measurements. Estimated GLUT-4 concentrations and GLUT-4 expression measured by Western blotting in the same biopsies were significantly correlated (r = 0.42, P = 0.02, n = 31).

**DISCUSSION**

By calculating an estimate of GLUT-4 immunoreactivity in slow and fast fibers in human skeletal muscle biopsies, we have demonstrated a fiber type dependency of GLUT-4 expression. On the basis of the dependency of insulin-stimulated glucose uptake and

<table>
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<th>Fiber Type</th>
<th>Diameter, µm</th>
<th>Density, nos./point</th>
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<tr>
<td></td>
<td>Slow fibers</td>
<td>Fast fibers</td>
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<tr>
<td></td>
<td>68.8 ± 2.6</td>
<td>70.8 ± 3.9</td>
</tr>
<tr>
<td>Nonstimulated</td>
<td>3.21 ± 0.09</td>
<td>2.84 ± 0.06</td>
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<tr>
<td>Stimulated</td>
<td>3.31 ± 0.12</td>
<td>2.85 ± 0.07</td>
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Values are means ± SE. Density was measured by counting nos. of grains/point. Significance was identified by Mann-Whitney test. There was no significant difference (NS) in density between basal and insulin-stimulated fibers.
GLUT-4 expression on fiber type in skeletal muscle from rodents (13, 17, 29, 34), a number of previous studies have considered such a relationship in human skeletal muscle (2, 24, 25, 38). GLUT-4 expression has been determined by Western blotting in homogenates or in membrane fractions of muscle biopsies and has been related to fiber type compositions, with inconclusive results (2, 24, 25). Nor could such a relationship be established in the present study. However, by using an alternative approach, immunohistochemistry combined with stereology, the localization and the number (density) of GLUT-4 immunoreactive sites within individual human muscle fibers could be determined. Our statistical analyses of this method show that it is excellent for the purpose of detecting small differences in GLUT-4 density among fiber types and between groups. That our finding of a higher number of immunoreactive sites in slow compared with fast muscle fibers reflects biological differences is supported both by the results from studies of rodents (13, 17, 29, 34) and by the significant correlation between the GLUT-4 content estimated morphometrically and that determined by Western blotting. The r value for this correlation is modest for several reasons. When GLUT-4 grains are measured, only clusters of GLUT-4 large enough to be registered are counted, and in addition, a grain is registered as one grain, regardless of its size. Estimates of GLUT-4 concentrations are based on fiber type volume fraction, which might vary along a muscle biopsy. Nevertheless, our results are in line with a published abstract in which GLUT-4 expression was determined by Western blotting of pooled, type-characterized, single human muscle fibers (38). Apart from our own approach to determining GLUT-4 in cryosections of human muscle, this is the only successful, however extremely laborious, demonstration of a fiber type-dependent GLUT-4 expression in human muscle.

The expression level of GLUT-4 is believed to be a determinant for the responsiveness (GLUT-4 expression) as well as the sensitivity (GLUT-4 translocation) of glucose uptake in insulin-sensitive cell systems. In human skeletal muscle, two of the stimuli for glucose uptake mediated by the GLUT-4 transporter are insul}

Table 3. Western blot. GLUT-4 expression in younger and older subjects

<table>
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<th>29 yr olds</th>
<th>64 yr olds</th>
<th>P</th>
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<tr>
<td>Nonstimulated</td>
<td>2.06 ± 0.63</td>
<td>1.37 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td>Stimulated</td>
<td>2.10 ± 0.38</td>
<td>1.36 ± 0.21</td>
<td>P &lt; 0.04</td>
</tr>
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Values are means ± SE expressed in optical density (OD)/mg tissue. There was no significant difference (by Mann-Whitney test) between basal and insulin-stimulated fibers.
lin and contraction. Both are believed to operate through a translocation mechanism (12, 28, 32). Type I muscle fibers are more insulin sensitive (29, 31, 47) than type II fibers, and type II fibers are more responsive to contraction-induced glucose uptake than type I fibers (41). In humans, such a relationship has been directly demonstrated by comparing insulin-stimulated glucose uptake of in vitro incubated human muscle strips with their fiber type composition (47). Whole body insulin-stimulated glucose uptake rate was, however, not correlated with slow fiber fraction in a muscle biopsy in some other studies (2, 25, 30, 31) and in the present one. Under the applied conditions, >80% of glucose uptake is directed to muscle (22). Fiber type composition is, however, only representative of parts of the biopsy itself. In a study by Dela et al. (6), whole body glucose uptake rates during euglycemic clamps at increasing insulin levels and GLUT-4 in the vastus lateralis were not correlated. Glucose uptake rates in the leg, representing skeletal muscle glucose uptake to a higher degree than whole body glucose uptake during the same clamp studies, were highly significantly correlated with GLUT-4 in the same biopsies of the vastus lateralis.

Ezaki et al. (10) determined the GLUT-4 content in quadriceps and soleus in young and older rats, and they found a reduced GLUT-4 content in white quadriceps from older rats. Our study of human muscle establishes a similar age-related decline in GLUT-4 expression level in type II fibers. Houmard et al. (26) described a negative association between age and insulin sensitivity as well as GLUT-4 content. Mice, where one allele of the GLUT-4 gene is disrupted in muscle and adipose tissue, develop insulin resistance and hyperglycemia (44). This impaired glucose uptake could be normalized by expressing GLUT-4 in fast-twitch muscles (45). Thus it could be expected that a reduction in fast-twitch GLUT-4 in humans could contribute to impaired insulin-stimulated glucose uptake and insulin resistance.

Age per se seems not to be a significant cause of insulin resistance (11). It could be speculated that decreased physical activity with increasing age might lead to a reduced GLUT-4 expression in muscle and consequently to a decreased potential of glucose uptake. In a study of athletes and a group with normal physical activity level, Melichna et al. (37) reveals an age-associated gain of type I fibers with a concomitant loss of type IIb fibers. Physical training retarded these age-related fiber changes (37). Electrically stimulated muscle contractions induce a shift of skeletal muscle fiber type from type IIb to type IIa in spinal cord injured patients (1). Denervation of hindlimb muscle decreases GLUT-4 expression in rats (15, 18, 36). In tetraplegic persons, electrically stimulated muscle contractions markedly improved insulin-stimulated glucose uptake through a major increase of key proteins involved in glucose metabolism, including GLUT-4, without changes in muscle fiber type distribution (21). Exercise increases skeletal muscle GLUT-4 in middle- and older-aged humans (8, 24, 25) and enhances whole body insulin sensitivity (7, 8, 16, 19, 24). Physical inactivity seems to be followed by reduced insulin sensitivity, reduced GLUT-4 levels, and a loss of type II fibers. Thus our finding of a reduced GLUT-4 expression, mainly in fast fibers, with age could be attributed to decreased physical activity.

In this study we have examined the GLUT-4 expression in individual human muscle fibers. GLUT-4 was expressed more abundantly in slow compared with fast fibers in both young and elderly subjects with reduced glucose tolerance. Furthermore, GLUT-4 expression decreased in fast muscle fibers with age. We hypothesize that variations in fiber type composition and GLUT-4 expression levels in human skeletal muscle might contribute to the interindividual variations in insulin-stimulated glucose uptake rates, and that one component of a multifactorial process involved in the development of age-related insulin resistance could be a reduced GLUT-4 expression in muscle, particularly in fast fibers, and possibly elicited by reduced physical activity.

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REFERENCES


Gundersen HJ and Jensen EB.

Hardin DS, Azzarelli B, Edwards J, Wigglesworth J, Mardt MA, and Holloszy JO. Evidence from transgenic mice that elevated skeletal muscle (Glut4) protein level.


