Comparison of GLUT1, GLUT3, and GLUT4 mRNA and the Subcellular Distribution of Their Proteins in Normal Human Muscle

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Basal, "insulin-independent" glucose uptake into skeletal muscle is provided by glucose transporters positioned at the plasma membrane. The relative amount of the three glucose transporters expressed in muscle has not been previously quantified. Using a combination of qualitative and quantitative ribonuclease protection assay (RPA) methods, we found in normal human muscle that GLUT1, GLUT3, and GLUT4 mRNA were expressed at 90 ± 10, 46 ± 4, and 156 ± 12 copies/ng RNA, respectively. Muscle was fractionated by DNase digestion and differential sedimentation into membrane fractions enriched in plasma membranes (PM) or low-density microsomes (LDM). GLUT1 and GLUT4 proteins were distributed 57% to 67% in LDM, whereas GLUT3 protein was at least 88% in the PM-enriched fractions. These data suggest that basal glucose uptake into resting human muscle could be provided in part by each of these three isoforms.

OF THE FIVE ISOFORMS of facilitative glucose transporters that have been cloned and characterized, three are involved in glucose uptake into skeletal muscle.[1-4] These are named GLUT1, GLUT3, and GLUT4 based on the order in which they were cloned.[1,4] GLUT2, the liver glucose transporter, is not expressed in muscle.[3] GLUT5 is expressed in muscle, but it only transports fructose.[5]

GLUT4, the insulin-responsive glucose transporter, is predominantly sequestered at an intracellular site in the basal state.[1,4] Insulin stimulates translocation of GLUT4 to the sarcolemma and transverse tubules, where GLUT4 facilitates glucose entry into the muscle cell.[6-8] GLUT1 and GLUT3 are thought to reside almost entirely on the cell surface, where they can function to provide insulin-independent, basal glucose uptake.[1,9,10]

In one rat muscle cell culture system, GLUT1, GLUT3, and GLUT4 were present on the cell surface in equal amounts,[11] but there has been no measurement of the amount of each of these transporters that normally reside on the human muscle cell surface. To obtain some indication of the relative contribution of these glucose transporters to the basal cell-surface glucose transporter pool, we quantified the amount of mRNA and the subcellular localization of the proteins for GLUT1, GLUT3, and GLUT4 in normal human skeletal muscle.

SUBJECTS AND METHODS

Subjects

Six normal subjects were recruited to participate in these studies. The protocol was reviewed and the consent document was approved by the University of Texas Medical Branch at Galveston (UTMB) Institutional Review Board. After written informed consent was obtained, each subject was admitted to the UTMB General Clinical Research Center. After an overnight fast and 2 hours of quiet supine bed rest, a percutaneous biopsy of the vastus lateralis muscle was obtained using a 5-mm Bergstrom-Stille biopsy needle. The 50- to 100-mg specimens were frozen in liquid nitrogen within 30 seconds. The 3 males and 3 females were within 20% of their ideal weight, had a normal 75-g oral glucose tolerance test, had no parents or siblings with diabetes, exercised less than 3 hours per week, and ranged in age from 22 to 42 years.

A reference sample used extensively in these studies was obtained from the gastrocnemius muscle at the autopsy of a young adult male trauma victim. This autopsy was performed 4 hours after unsuccessful resuscitation. This specimen contained strong and consistent signals for GLUT1, GLUT3, and GLUT4 mRNA and protein. There were no extraneous bands in the immunoblots or ribonuclease protection assays (RPAs) that might suggest significant degradation.[12]

Materials

The American Type Culture Collection (ATCC) provided cDNAs for human GLUT1, human GLUT3, and human GLUT4. Glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was purchased from Ambion (Austin, TX). pBSM13 was purchased from Stratagene (La Jolla, CA). Affinity-purified antisera against human GLUT1, GLUT3, and GLUT4 were purchased from Alpha Diagnostic International (San Antonio, TX). Riboprobe Gemini II Core System kits were purchased from Promega (Madison, WI). α-^{32}P-UTP 800 Ci/mmol was purchased from DuPont NEN (Boston, MA). Vanadyl Ribonucleoside Complex was purchased from GIBCO-BRL (Grand Island, NY). Ribonuclease T1 was purchased from Sigma (St. Louis, MO). Ribonuclease A and DNase were purchased from Boehringer-Mannheim (Indianapolis, IN). All other chemicals were reagent grade.

RNA Isolation

Total cellular RNA was isolated from skeletal muscle using the Chomczynski-Sacchi[13] RNAzol B method (Tel-Test, Friendswood, TX) with 2 mL RNAzol B/100 mg muscle. Twelve consecutive preparations averaged 0.8 µg RNA/mg muscle.

Production of Riboprobes

Riboprobes for each GLUT were prepared by cutting a piece of cDNA from the ATCC-provided plasmid and ligating it into pBSM13 as described previously.[12] These pieces, 320 bp for GLUT1, 256 bp for GLUT3, and 650 bp for GLUT4, were from the middle of the glucose transporter cDNA and spanned 4 to 6 exons. Each was inserted into the plasmid oriented such that the T3 RNA polymerase resulted in an antisense riboprobe. Transcription was performed with 10 mmol/L

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dithiothreitol, 0.5 mmol/L rNTPs without UTP, 40 μmol/L UTP, 200 ng linearized template DNA, 50 μCi α-^{32}P-UTP, and 1 μL T3 RNA polymerase for 30 minutes at 37°C. Vanadyl Ribonucleoside Complex RNase inhibitor (1 μL), 1 μg DNase I, and 8 μg tRNA were added and the incubation was continued for another 15 minutes. The labeled probe was precipitated with ethanol, vortexed, washed, dried, and resuspended in water.

The pTRI-GAPDH plasmid (Ambion) contained a 316-bp fragment of the GAPDH ''housekeeping'' gene. This plasmid was used to generate an internal standard for the RPA. Digestion with *Dde*I yielded a probe that protected a 154-base RNA fragment. The choice of 316 or 154 bases as the size for the GAPDH probe is determined by the size of the protected bands for the other probes. An antisense riboprobe of GAPDH was constructed following the riboprobe transcription reaction already described.

RPA

The RPA was performed by the method of Lowe et al[14] with minor modifications as previously described.[12] Briefly, RNA samples for assay were resuspended in hybridization buffer (80 mmol/L Tris, 4 mmol/L EDTA, 1.6 mol/L NaCl, and 0.4% sodium dodecyl sulfate [SDS], pH 7.5), and 1 to 2 μL of the labeled riboprobe was added. Incubation was performed at 85°C for 5 minutes and then at 45°C for 15 to 20 hours. Ribonucleases T1 (54 U) and A (40 mg/mL final) were added, and after 1 hour at 30°C, the reaction was stopped by adding SDS and proteinase K. Electrophoresis was performed on an 8-mol/L urea, 8% polyacrylamide gel after denaturing the sample at 95°C for 3 minutes.

Each dried gel was analyzed using images from a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Band intensities were quantified using the ImageQuant software provided with the PhosphorImager. The intensities of protected bands were corrected by GAPDH intensity as a control for total RNA variability. The GLUT3/GLUT4 RPA was optimized for linearity over a range of 5 to 30 μg per lane. Reproducibility of the quantification of GLUT4 was indicated by a variance of 8.4% (SD) using the same muscle preparation in 6 separate lanes. When corrected for GAPDH band intensity, the variance decreased to 4.8%. Intensity was quantified with an arbitrary scale determined in each RPA using RNA from the reference sample described earlier.

Quantitative RPA for GLUT1, GLUT3, and GLUT4

The RPA was performed as described before, except that the sample RNA was added at an increasing dose, as much as 100 μg/lane, such that a clear excess of sample RNA relative to probe was achieved at the higher doses. The specific activity of each ^{32}P-labeled probe was measured,[15] and the phosphor image was calibrated with known cpm amounts of probe such that the image analysis software could quantify the cpm of probe represented by a specific band. The amount of labeled probe was kept constant and the total RNA added per tube was increased until well above the point where excess unlabeled mRNA was achieved. Protected, labeled probe counts per lane increased until the unlabeled mRNA was in excess of the labeled probe. The point of inflection where the addition of more unlabeled mRNA did not further increase the protected counts determined the maximum hybridization efficiency and the moles of mRNA per nanogram total RNA. Determining these numbers using the quantitative RPA technique requires a large amount of RNA (100 to 150 μg per experiment) from the muscle specimen and therefore is not practical using the standard percutaneous needle muscle biopsy specimen directly. The absolute copy number of mRNA in normal control subjects was determined by multiplying the absolute amount determined in the reference sample by the protected band intensity for each subject relative to the reference sample included in each RPA.

Membrane Preparation

The techniques we have developed are based on the method of Hirshman et al[16] and involve sharp blade homogenization in 0.25 mol/L sucrose, broad protease inhibition, DNase digestion, and differential sedimentation. Briefly, 50 mg muscle was finely minced and homogenized on ice in 1 mL buffer A (20 mmol/L HEPES, 250 mmol/L sucrose and 1 mmol/L EDTA, pH 7.4, containing 1 mmol/L PMSF, aprotinin 0.2 μg/mL, leupeptin 0.2 μg/mL, and pepstatin 2 μg/mL) for 20 seconds using a small-probe Polytron homogenizer (Brinkman, Westbury, NY). A 300-μL volume of 10× buffer B (3 mol/L KCl and 250 mmol/L sodium pyrophosphate) was added and the volume was increased to 3 mL with buffer A. The homogenate was centrifuged at 227,000 × *g* for 60 minutes. The pellet was resuspended in 675 μL buffer A, 125 μL DNase (80,000 U/mL) was added, and the sample was incubated for 60 minutes at 30°C. The sample was centrifuged again at 227,000 × *g* and the pellet resuspended in 1 mL buffer A. The sample was then centrifuged at 12,000 × *g* for 15 minutes, the supernatant was saved as low-density microsomes (LDM), and the pellet was resuspended in 250 μL buffer A and labeled plasma membrane (PM-enriched). Protein content was measured using the Bradford method.[17] The protein yield for 14 consecutive 50-mg muscle preparations was 6,400 ± 440 μg LDM and 1,000 ± 190 μg PM. 5′-Nucleotidase activity[18] in PM fractions was increased 5-fold relative to the homogenate and was 0.7 of the homogenate activity in LDM fractions. Galactosyl transferase activity[19] was increased 8-fold in LDM compared with homogenate. Each gel lane for Western blots used 5 to 80 μg membrane protein depending on the fraction and the isoform to be assayed.

PM-enriched fractions contain both sarcolemma and transverse tubules. Transverse tubules cannot be separated from sarcolemma solely by differential sedimentation because their equilibrium densities are very similar in the absence of the addition of calcium phosphate, as reported by Rosemblatt et al.[20] In this report, we use the term muscle cell PM to specifically include both the sarcolemma and the transverse tubules.

Immunoblots

Muscle membranes were subjected to 12% SDS-polyacrylamide gel electrophoresis[21] and electrophoretically transferred to nitrocellulose filters. The affinity-purified anti-human GLUT1, GLUT3, and GLUT4 antisera were generated using keyhole limpet hemocyanin-fixed synthesized peptides that correspond to the carboxy-terminal 12 amino acids from the human glucose transporters. The antisera were used at final dilutions of 1:750 to 1:2,000. The filters were blocked with 8% nonfat dry milk in TBS (50 mmol/L TRIS, 138 mmol NaCl, 2.7 nmol/L kCl, pH 7.4) with 0.4% Triton for at least 2 hours, washed, and incubated with primary antibody for 2 hours at 20°C. The filters were washed again and incubated with secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase, diluted 1:2,500 in 1% dried milk/TBST) for 1 hour. The filters were washed 5 times and finally developed by an enhanced chemiluminescent assay using an ECL Kit from Amersham Pharmacia Biotech (Piscataway, NJ). Dried filters were exposed using XAR-5 film (Eastman Kodak, Rochester, NY) for 5 seconds to 3 minutes. Nonspecific labeling was determined by omitting the primary antibody. Autoradiograms were digitized and analyzed using the ImageQuant software provided with the PhosphorImager.

RESULTS

Molar Ratios of GLUT1, GLUT3, and GLUT4 mRNA in Normal Human Skeletal Muscle

Quantification of the number of copies of the mRNAs for GLUT1, GLUT3, and GLUT4 was performed in total RNA extracted from a reference preparation of human skeletal

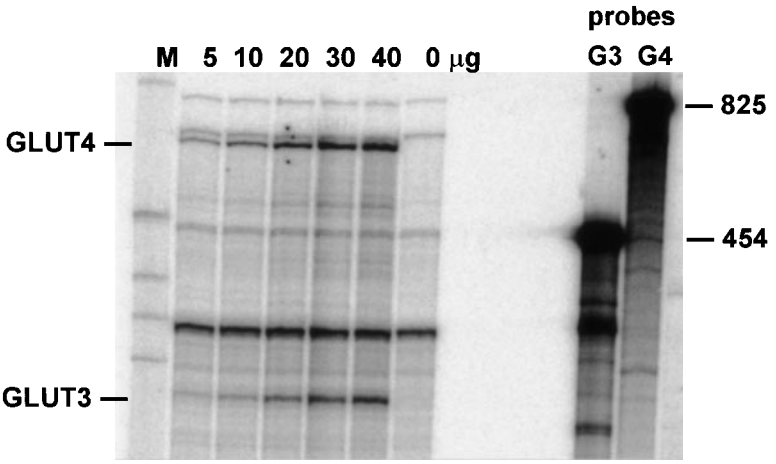

Fig 1. Quantitative RPA of GLUT3 and GLUT4 mRNA from a reference muscle RNA preparation. The riboprobes for GLUT3 and GLUT4 are designated G3 and G4. M designates the lane containing molecular weight markers, and RNA content is shown at the top of each lane (5-40 μg and 0 μg). The specific protected bands for GLUT4 and GLUT3 are indicated on the left. The lane labeled "0 μg" contained no RNA from muscle and the bands represent nonspecific bands related to the probes only. The image analysis calculations subtracted the background determined in this lane from the band intensities at the corresponding size locations.

muscle as described earlier. These numbers then were used in calculating the number of copies in muscle from 6 normal control subjects, based on the expression relative to the reference sample that was included in each RPA. The reference muscle was used in 8 separate experiments to measure the number of molecules of GLUT3 and GLUT4 message per nanogram muscle tissue RNA. Figure 1 shows a phosphor image of one of the experiments for GLUT3 and GLUT4 mRNA quantification. GLUT1 molar quantification in the reference muscle specimen was determined in 4 separate experiments, and Fig 2 shows a phosphor image of one of those. GLUT1 experiments were separate from GLUT3 and GLUT4 studies because the GLUT1 protected-band apparent molecular weight was similar to that of the probe for GLUT3. The reference muscle preparation was determined to have 85 ± 6 copies of GLUT1 mRNA/ng RNA, 100 ± 14 copies of GLUT3 mRNA/ng RNA, and 96 ± 18 copies of GLUT4 mRNA/ng RNA. The data from 6 normal subjects are shown in Fig 3. Even though the reference sample had very similar amounts of each of the three principal transporters, the normal subjects had a ratio of 2:1:3 for GLUT1, GLUT3, and GLUT4.

Subcellular Distribution of GLUT1, GLUT3, and GLUT4 Proteins in Normal Human Muscle

Muscle obtained by needle biopsy from 6 normal control subjects was frozen in liquid nitrogen within 1 minute of the time the biopsy was taken from the subject. Each subject fasted at least 12 hours and was maintained quietly supine for at least 2 hours prior to performing the biopsy. Each specimen was thawed at a later time and fractionated, and membrane fractions were immunoblotted as described earlier. Figure 4 displays a representative immunoblot of muscle membrane fractions from 6 control subjects and our reference muscle specimen probed with the affinity-purified, anti-hGLUT1 polyclonal antibody. Figure 5 displays the percent of the total immunoreactivity

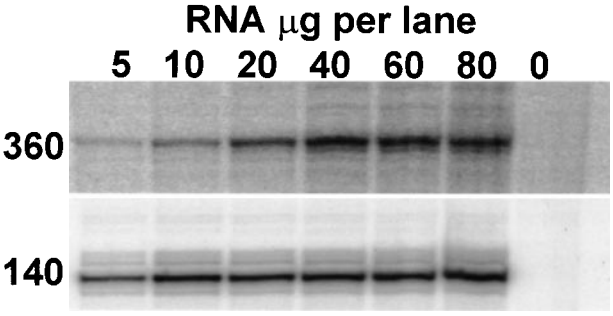

Fig 2. Quantitative RPA of GLUT1 mRNA. The same reference muscle specimen used for GLUT3 and GLUT4 mRNA quantification was also used in similar studies for determining the absolute concentration of GLUT1 mRNA. This phosphor image is typical of 4 separate studies used to calculate the number of copies of GLUT1 mRNA in the reference specimen. Numbers on the left represent the apparent size in bases of the protected bands on this gel. The band at 360 is GLUT1 and the band at 140 is GAPDH. For the GAPDH component of the gel, this image had the overall intensity adjusted 10-fold less than that shown for the GLUT1 protected band. The reference sample was determined to have 85 ± 6 copies of GLUT1 mRNA per nanogram total RNA. GAPDH mRNA was determined to be 12,600 ± 1,990 copies per 1 ng RNA in the reference muscle specimen in a total of 10 studies.

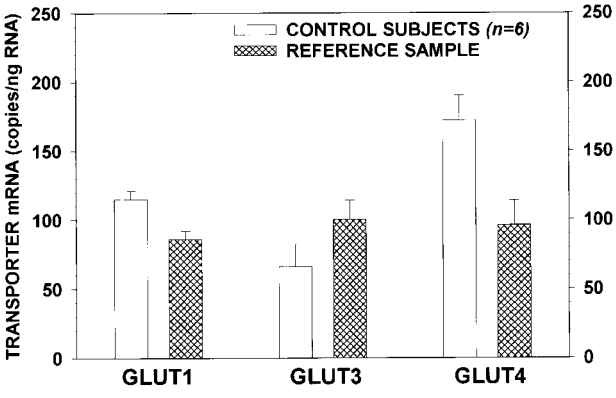

Fig 3. Glucose transporter mRNA copy number in normal human muscle. The data represent the quantification of GLUT1, GLUT3, and GLUT4 mRNA copy number per nanogram RNA for our reference muscle sample and 6 control subjects. The reference sample data were determined using the quantitative RPA technique. Each control subject's glucose transporter message amount was determined initially in at least 2 separate RPAs expressed relative to the reference muscle specimen signal and then converted using the copy number determined for the reference sample. The data shown for the reference sample are the mean ± SD from 4 experiments for GLUT1 and 8 separate studies for GLUT3 and GLUT4.

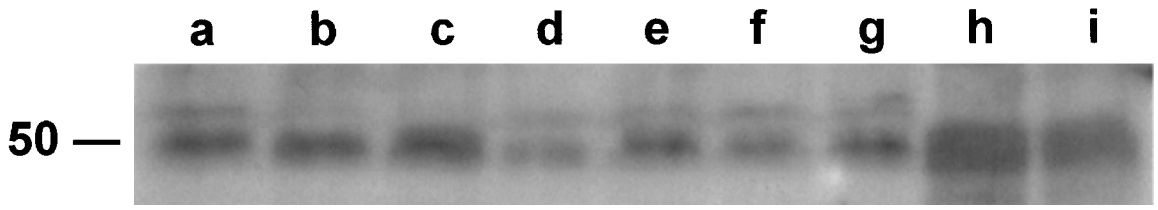

Fig 4. Immunoblot of skeletal muscle membranes probed with an anti-GLUT1 antibody. Shown is a film image from a typical immunoblot. Lanes a through g contain 40 μg PM-enriched fractions and lanes h and i contain 80 μg Golgi-enriched fractions. Lanes a and h are fractions from our reference muscle specimen, whereas lanes b through g represent 6 different normal subjects. Lanes e and i are from the same control subject.

measured in the two fractions for GLUT1, GLUT3, and GLUT4. The total immunoreactivity in PM and LDM fractions is calculated by multiplying the band intensity expressed in arbitrary units per microgram applied to the gel times the total micrograms of protein yield for the respective fraction. Each pair of bars represents data from 6 individuals analyzed in at least two experiments. These data show that GLUT3 protein is almost entirely PM-associated, whereas GLUT1 and GLUT4 are predominantly found in the Golgi-enriched fractions.

DISCUSSION

The data presented in this communication provide the first quantification of the amount of the three glucose transporter mRNAs present in human muscle. We found that normal human skeletal muscle contains GLUT1, GLUT3, and GLUT4 mRNA at a molar ratio of about 2:1:3. A determination of the absolute protein concentration of each transporter could not be made in these studies, but the subcellular distributions of the proteins were compared. GLUT1 and GLUT4 proteins are similarly distributed in muscle in the basal state, with 33% to 43% of these two glucose transporters being found in the PM-enriched fractions of muscle specimens fractionated by DNase digestion and differential sedimentation. The PM-enriched fractions contained 88% of the GLUT3 protein.

Human skeletal muscle contains mRNA for GLUT1, GLUT3, and GLUT4. Whether the amounts of GLUT1 or GLUT3 present are physiologically important in man has been questioned.[3] GLUT1 is present at the cell surface in rat muscle and was considered to mediate basal glucose uptake.[1] GLUT3 is expressed most abundantly in brain and neural tissue. Its presence in muscle has been attributed by some investigators to a contamination of muscle specimens with the nerves that innervate the fascicles.[22] Perineural cells contained in muscle biopsies display strong signals for GLUT3 mRNA by in situ hybridization and GLUT3 protein and by immunohistochemistry, but nearly 90% of the signals are found in muscle cells.[23] A similar pattern of GLUT1 distribution as determined by immunofluorescence was shown by Handberg et al[24] in rat muscle. They found a strong GLUT1 signal in perineural cells, but none in vascular endothelium.[24] However, this is in contrast to the brain, where GLUT1 is expressed in both endothelial cells and glial cells.[25] Similar to reports of the distribution of GLUT4 and GLUT1 protein in rat muscle,[26] we found that GLUT3 was expressed at higher levels in type 1 oxidative (slow-twitch) fibers.[23]

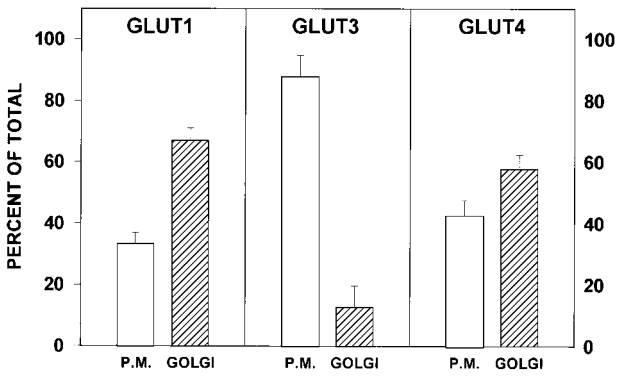

Fig 5. Subcellular distribution of glucose transporters in normal human muscle. Skeletal muscle obtained by needle biopsy from normal individuals was subjected to subcellular fractionation and quantified by image analysis of immunoblot films. Each pair of bars represents the percentage of the immunologic signal attributable to the entire PM-enriched fraction or LDM. The determination was performed at least twice for each transporter for each subject. The data for GLUT1 represent 4 subjects, and the data for GLUT3 and GLUT4 represent 6 subjects.

We speculate that previous reports of undetectable GLUT3 mRNA[27,28] or protein[29,30] in human muscle were due to the assay of muscle samples from autopsies containing degraded mRNA and protein. We have recently shown that several mRNA species are rapidly degraded in muscle at room temperature and that GLUT3 protein disappears at an even faster rate than GLUT3 mRNA.[12] Muscle specimens obtained at autopsy are thus likely to dramatically underestimate the quantity of mRNA or protein that is actually present in normal muscle tissue.

The primary data reported here include the number of copies per nanogram total RNA of each glucose transporter mRNA in normal muscle and the subcellular distribution of the corresponding proteins. Since there are no published quantitative immunoblot methods for estimation of the absolute amount of glucose transporter proteins, the amounts of different GLUT proteins cannot be directly compared. There have not been any published attempts at quantification of mRNA or protein in vivo turnover rates for glucose transporters. Realizing that the following estimates of glucose transporter pool composition are highly speculative, nevertheless, if one assumes that the ratio of protein to its message concentration is similar among these three transporters, a calculation can be made. The basal plasma membrane glucose transporter pool would be 25% GLUT1, 45% GLUT3, and 30% GLUT4. Similar calculations would set the composition of the intracellular pool of transporters at 33% GLUT1, 4% GLUT3, and 63% GLUT4. Even if 12% of the GLUT3 in the sample is attributed to contamination with neural elements,[23] GLUT3 is still the most numerous at the cell surface

under the conditions that we examined. As much as 60% of GLUT1 protein may be associated with perineural cells,[24] thus further increasing the GLUT3 relative proportion in the cell surface pool of glucose transporters. Burdett et al[31] isolated PM and transverse tubules from rabbit skeletal muscle and demonstrated by cytochalasin-B binding that most glucose transporters were in the transverse tubule fractions. Using immunogold and electron microscopy, GLUT4 protein has been estimated to be 10% or less at the sarcolemma, the majority associated with the sarcoplasmic reticulum, and a portion was associated with transverse tubules and triads.[6,7] The muscle membrane fractionation method that we used in these studies results in significant cross-contamination of the PM-enriched fraction with Golgi membranes and vice versa. Thus, the PM-enriched fraction GLUT1 and GLUT4 protein content is probably an overestimate. Further, GLUT1 protein in each muscle biopsy specimen may contain a contribution from red blood cell membranes. This potential contamination is likely to be fairly consistent among specimens, and comparisons among groups of subjects may still be made.

The amount of GLUT1 and GLUT4 mRNA in the reference muscle specimen was lower than in the normal control subjects. The medical history of the trauma victim is unknown. Further, the specimen came from a different muscle group, the gastrocnemius rather than the vastus lateralis as in the controls. There is also a potential for degradation-related underestimation of mRNA concentrations in the reference specimen.[12]

The actual composition of these glucose transporter pools must await absolute protein data. Holman et al[32] quantified the ratio of GLUT4 to GLUT1 in isolated rat adipocyte PM using a photolabeled bis-mannose followed by precipitation with separate antibodies specific to GLUT4 or GLUT1. These studies assumed equal binding of the photolabel and equal immunoprecipitation by the different antibodies. Their data suggested that GLUT4 comprised about two thirds of the basal cell-surface glucose transporters. In response to maximal insulin stimulation, GLUT4 on the cell surface increased 25-fold and GLUT1 increased about 4-fold, with GLUT4 thus accounting for 90% of the fat cell-surface glucose transporters in the insulin-stimulated state. These investigators did not use any anti-GLUT3 antibody, but they did identify a small amount of specific photolabeling that did not immunoprecipitate with either GLUT1 or GLUT4 antibody.[32] Their basal GLUT1/GLUT4 ratio was 0.4/1.0 to 0.7/1.0, depending on the order of immunoprecipitation. The only direct comparison of GLUT1, GLUT3, and GLUT4 expression performed previously was reported by Wilson et al[11] in cultured cells. They directly compared the cell-surface protein content of GLUT1, GLUT3, and GLUT4 using a radioactive photolabel and specific immunoprecipitation,[11] similar to the methods used by Holman et al.[32] In myotubes from the rat muscle-derived L6 cell line, they found that the amount of GLUT1, GLUT3, and GLUT4 on the cell surface in the absence of insulin was 14.2, 9.7, and 11.6 fmol/mg muscle protein, respectively.[11] Using subcellular distribution data previously published by this group,[33] one can estimate that the total cellular protein for these transporters was about 26, 16, and 28 fmol/mg muscle protein, respectively. Expressing the protein for GLUT1 and GLUT3 relative to GLUT4 from their data yielded a ratio of 0.9/0.6/1.0 for GLUT1/GLUT3/GLUT4 protein in unstimulated L6 myotubes. Our data for mRNA in normal human muscle were similar at 0.6/0.3/1.0 for GLUT1/GLUT3/GLUT4.

By virtue of its lower K_m for D-glucose,[34] GLUT3 could be the glucose transporter normally responsible for more than half of the basal glucose uptake in human muscle.

The data presented in this report provide the first actual number of copies of mRNA for each of the principal glucose transporters in normal human muscle. In addition, the relative amounts of each of the corresponding proteins on the cell surface or inside the cell were estimated. These quantitative data suggest that all three of these glucose transporters (GLUT1, GLUT3, and GLUT4) are involved in basal glucose uptake into muscle, and further, GLUT3 may play a more important role than previously appreciated.

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