GLUT3 Protein and mRNA in Autopsy Muscle Specimens

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GLUT3 is expressed in rat muscle, but this glucose transporter protein has not been identified previously in adult human skeletal muscle. We quantified the rapidity of disappearance of mRNA and protein from human skeletal muscle at room temperature and at 4°C. Fifty percent of the immunologically detectable GLUT3 protein disappeared by 1 hour at 20°C and by 2 hours at 4°C. mRNA for GLUT3 was decreased 50% by 2.2 hours at 20°C and by 24 hours at 4°C. Half of the measurable mRNAs for GLUT4, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), α-actin, and β-myosin disappeared by 0.8 to 2.1 hours at 20°C and by 5.0 to 16.6 hours at 4°C. Previous conclusions that GLUT3 is not expressed in human muscle were likely drawn because of artifacts related to degradation of GLUT3 protein in the specimens prior to study. Because of the rapid degradation of protein and mRNA, autopsy specimens of muscle must be obtained within 6 hours of death, and even then, protein and mRNA data will likely dramatically underestimate their expression in fresh muscle. Some previously published conclusions and recommendations regarding autopsy specimens are not stringent enough to consistently yield useful protein and mRNA.

THREE GLUCOSE TRANSPORTER isoforms (GLUT1, GLUT3, and GLUT4) are involved in glucose uptake into human skeletal muscle.[1,2] The role of GLUT3 in skeletal muscle has not been clear in previous publications.[1,3] We found strong GLUT3 signals in muscle obtained by needle biopsy from normal control subjects (unpublished observations), but weak to absent signals in many autopsy samples. Gould and Bell[4] suggested that both GLUT1 and GLUT3 mRNA levels are very low in muscle and that other transporters must be responsible for muscle glucose uptake. Klip and Paquet[5] suggested that GLUT1 was the principal muscle transporter providing basal glucose uptake. Gould and Holman[3] have suggested that GLUT3 may only be found in muscle by virtue of contamination of muscle samples with neural tissue. Unfortunately, there have been few studies of GLUT3 expression in human muscle, and the data from some of these are difficult to compare because of differing time intervals and processing methods.

Samples of fresh human tissues are not readily available. It is common to use autopsy material as a source of human tissue of adequate quantity for isolation of mRNA or the protein product from a gene of interest. This is particularly true of early descriptive studies of the relative amount of gene expression in different tissues. Studies of glucose transporter expression are a good example of this approach, where extensive investigations were performed in rats and correlations were then attempted in human tissues.[6-10] Several early studies of human mRNA or protein immunoblots used autopsy material as a source of RNA or protein.[6,7,9] Some used diagnostic surgical biopsies,[10] and at least one group used muscle from the amputated legs of diabetic patients.[11] Most of these descriptive studies did not provide details about the time from death to autopsy or the disease that caused death.[8] Haber et al[9] evaluated glucose transporter mRNA quantification in various tissues obtained from cadavers up to 24 hours after death. They found no detectable GLUT3 mRNA in four muscle specimens. Shepherd et al[8] were able to identify GLUT3 mRNA but could not detect GLUT3 protein by immunoblot.

To set up methods for analysis of glucose transporter mRNA and immunoblots, we obtained muscle specimens from autopsies within 24 hours of death. We found that the amount of specific mRNAs was variable, but the amount of usable RNA generally declined with an increasing interval between death and obtainment of tissue samples. The time course of the disappearance of quantifiable mRNA and protein for GLUT3, determined by incubation at room temperature and at 4°C, showed rapid degradation for both. Protein loss was more rapid than mRNA disappearance. The rapidity of degradation of GLUT3 protein and mRNA suggests that the maximum acceptable time between death and procurement of tissues at autopsy needs to be much shorter.

MATERIALS AND METHODS

Materials

The American Type Culture Collection ([ATCC] Rockville, MD) provided cDNAs for human GLUT3 and human GLUT4. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was purchased from Ambion (Austin, TX). pBSM13 was purchased from Stratagene (La Jolla, CA). Antisera against human GLUT3 and GLUT4 were purchased from Charles Rivers East Acres Biologicals (Southbridge, MA). Riboprobe Gemini II Core System kits were purchased from Promega (Madison, WI). α-^{32}P-uridine triphosphate (UTP) 800 Ci/mmol was purchased from DuPont NEN (Boston, MA). Vanadyl Ribonucleoside Complex (VRC) was purchased from GIBCO (Grand Island, NY). RNase T1 was purchased from Sigma (St Louis, MO). RNase A and DNase were purchased from Boehringer (Indianapolis, IN). All other chemicals were reagent-grade.

RNA Isolation

Total cellular RNA was isolated from skeletal muscle with the method of Chomczynski and Sacchi[12] using RNAzol B (Tel-Test, Friendswood, TX) at a concentration of 2 mL/100 mg muscle. Twelve consecutive preparations had an average of 0.8 μg RNA/mg muscle.

Membrane Preparation

The technique we have developed is based on the method of Hirshman et al,[13] and involves sharp-blade homogenization in 0.25 mol/L sucrose, DNase digestion, and differential sedimentation. Briefly,

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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 phenylmethylsulfonyl fluoride, 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). Three hundred microliters of 10× buffer B (3mol/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 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 saved as low-density microsomes (LDMs), and the pellet resuspended in 250 μL buffer A and labeled as plasma membrane–enriched (PM). 5′-Nucleotidase activity[14] was increased fivefold in PM fractions relative to the homogenate and was 0.7 of the homogenate in LDM fractions. Protein content was measured using the Bradford method.[15] The protein yield for 14 consecutive 50-mg muscle preparations was 6,400 ± 440 μg LDM and 1,000 ± 190 μg PM. Each gel lane for Western blots used 5 to 80 μg membrane protein, depending on the fraction and the isoform to be assayed.

Production of Riboprobes

GLUT3 riboprobe. A 256–base pair (bp) *Sac*I/*Eco*RI GLUT3 cDNA fragment was isolated from pBSMGT3. This piece spans transmembrane segments M7, M8, and M9 and includes part of exons 6, 7, 8, and 9. The fragment was gel-purified and ligated into pBSM13 previously digested with *Sac*I and *Eco*RI. The plasmids were sequenced to check for correct insertion into pBSM13. Linearization of the construct with *Pvu*I and subsequent ethanol precipitation were performed prior to generation of the riboprobe. The transcription reaction was performed with 10 mmol/L dithiothreitol, 0.5 mmol/L ribonucleotide triphosphates 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. VRC RNase inhibitor (1 μL), 1 μg DNase I, and 8 μg tRNA were added and the incubation continued another 15 minutes. The reaction mixture was precipitated with ethanol, vortexed, washed, dried, and resuspended in water.

GLUT4 riboprobe. A 650-bp *Sac*I/*Xba*I GLUT4 cDNA fragment was isolated from pSPGT4. The fragment was then gel-purified and ligated into pBSM13. The GLUT4 riboprobe was generated by the same procedures used for the GLUT3 riboprobe.

GAPDH riboprobe. The pTRI-GAPDH plasmid (Ambion) contained a 316-bp fragment of the GAPDH "housekeeping" gene. This plasmid was used as an internal standard for the RNase protection assay (RPA). An antisense riboprobe of GAPDH was constructed following the riboprobe transcription reaction already discussed.

α-Actin riboprobe. Dr Laurence Kedes (University of Southern California, Los Angeles, CA) provided cDNA for human α-actin. This cDNA was ligated into pBSM13 for generation of riboprobes as already described for GLUT3. Probes were labeled as described earlier, and RPA was performed in a similar fashion as for GLUT3 and GLUT4 except that the amount of RNA subjected to hybridization was reduced to 2 μg.

β-Myosin riboprobe. Human β-myosin cDNA was obtained from the ATCC. The β-myosin cDNA was also inserted into pBSM13, and the probes were generated and assays performed as described for α-actin.

RPA

The RPA was performed by the method of Lowe et al[16] with minor modifications. Briefly, RNA samples to be assayed were precipitated in 0.3 mol/L sodium acetate and 2 vol 100% ethanol for 2 hours at −70°C. The RNA sample was 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 labeled riboprobe was added. This mixture was incubated for 5 minutes at 85°C and then for 15 to 20 hours at 45°C. RNases 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. The reaction mixture was extracted with phenol and chloroform and precipitated with ethanol. Before electrophoresis on an 8-mol/L urea, 8% polyacrylamide gel, the precipitate was suspended in sample buffer and denatured at 95°C for 3 minutes.

Each dried gel was analyzed using images from a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Band intensity was quantified using the ImageQuant software provided with the PhosphorImager. The intensity of protected bands was corrected by GAPDH intensity as a measure of total RNA recovery. The GLUT3/GLUT4 RPA was optimized for linearity over a range of 5 to 30 μg per lane. The reproducibility of GLUT4 quantification was indicated by a variance of 8.4% (standard deviation) using the same muscle preparation in six 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 muscle RNA from an autopsy specimen from a young adult male trauma victim (reference muscle sample).

Immunoblots

Muscle membranes prepared by a modification (already described) of the method of Hirshman et al[13] were subjected to 12% SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose filters. The anti–human GLUT3 and GLUT4 antisera (Charles River East Acres Biologicals) were generated using keyhole limpet hemocyanin–fixed synthesized peptides that correspond to the carboxy-terminal 12 amino acids from human GLUT3 and GLUT4 transporters. The antisera were used at a final dilution of 1:750 to 1:2,000. Filters were blocked with 8% nonfat dry milk in Tris-buffered saline (TBS) and 0.4% Triton for at least 2 hours, washed, and then incubated with primary antibody for 2 hours at 20°C. The filters were washed again and incubated with secondary antibody (goat anti–rabbit immunoglobulin G coupled to horseradish peroxidase, diluted 1:2,500 in 1% dried milk/TBS-Tween 20 [TBST]) for 1 hour. The filters were washed five times and finally developed by an enhanced chemiluminescence (ECL) assay using an ECL kit from Amersham (Piscataway, NJ). Dried filters were exposed using XAR-5 film (Eastman Kodak, Rochester, NY) for 5 seconds to 3 minutes.

RESULTS

Time Course of mRNA Degradation in Human Skeletal Muscle Obtained at Autopsy

To determine how quickly mRNA becomes undetectable by our riboprobes, a reference sample was incubated in multiple aliquots for 1 to 24 hours at either 20°C or 4°C. The reference sample was from the gastrocnemius muscle of a young male head-trauma victim in whom the autopsy occurred at 4 hours after unsuccessful resuscitation in the Emergency Department. Although this specimen was clearly not as fresh as the 50- to 150-mg specimens obtained from normal subjects, the yield of RNA and the pattern of glucose transporter proteins on immunoblots were similar. Thus, this specimen has been used extensively as a reference sample for RNA and immunoblot studies in our laboratory. Figure 1 displays images from a RPA that included samples incubated at 4°C for up to 24 hours. Control (freshly thawed) samples were used in the first two lanes and the last lane, indicated as 0 hours of incubation. Figure 2 displays

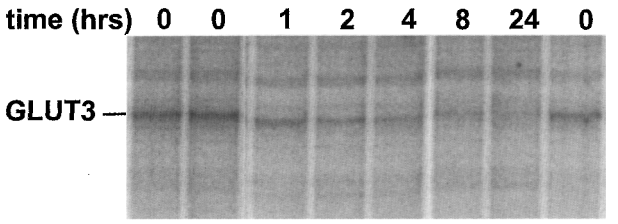

Fig 1. RPA for GLUT3 using RNA isolated from the reference muscle specimen at the indicated time (hours) of incubation at 4°C. This image is typical of 2 separate RPAs performed to analyze the samples incubated at either 4°C or 20°C.

the data for GLUT3 and GLUT4 mRNA detection by RPA with incubation at 4°C and 20°C.

Similar evaluations for the detection of GAPDH, α-actin, and β-myosin were performed in two separate experiments at each temperature for actin and myosin and in four separate experiments for GAPDH. A pattern similar to that for GLUT3 and GLUT4 mRNA disappearance was found. Table 1 lists the T_{50} (time at which the decline in the signal reached 50% of the control signal) for each of these time courses.

Recovery of GLUT3 Protein in Muscle Specimens From Autopsies

Muscle obtained from normal control subjects by percutaneous needle biopsy provided a single band at M_r 45,000 in immunoblots of PM fractions, whereas autopsy specimens obtained within 24 hours of death show one to three bands with an apparent molecular weight of 45, 42, and 35 kd (Fig 3). The pattern exhibited by these four autopsy specimens suggests that the GLUT3 protein is initially cleaved at two points near the amino terminus, since the antibody used is specific for the 12 amino acids at the carboxy terminus.

Time Course for Disappearance of Immunoblot Detection of GLUT3 Protein in Human Skeletal Muscle

In studies performed in parallel to the mRNA degradation experiments, 50-mg aliquots of the reference muscle were incubated up to 24 hours at either 4°C or 20°C, and the PM subcellular fractions were subjected to electrophoresis and

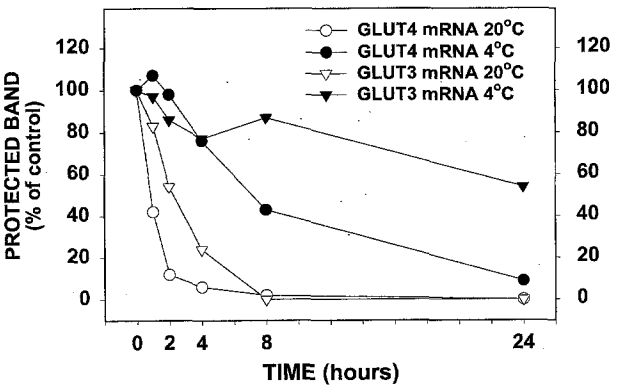

Fig 2. Time course for disappearance of GLUT3 and GLUT4 protected bands in a RPA. Data points represent the mean of 2 separate assays of samples incubated 1-24 hours at either 4°C or 20°C.

Table 1. T_{50} for Specific mRNA Species in Human Skeletal Muscle

mRNA	T_{50} (h) 20°C	4°C
GLUT3	2.2	24
GLUT4	0.8	7.2
GAPDH	2.1	16.6
α-Actin	0.8	7.3
β-Myosin	0.8	5.0

Abbreviation: T_{50}, incubation time required for RPA signal to decrease to 50% of the starting value.

immunoblotting as described in the Methods. Figure 4 displays the time-related decline in immunologically detectable GLUT3 protein at both temperatures. These data represent the relative intensity of the 45-kd band. At 4°C, the smaller 35-kd band was not present at time 0, but at 1 hour of incubation, it was equivalent to 8% of the time 0 control signal, 18% at 2 hours, 22% at 4 hours, 20% at 8 hours, and 2% at 24 hours.

DISCUSSION

Measurements of GLUT3 mRNA and protein content in human muscle obtained at autopsy have been reported to show either no mRNA or protein[6,9] or some mRNA but no protein.[8] We also found erratic results for such studies in a series of autopsy-derived muscle specimens, in contrast to the strong signals for both protein and mRNA in fresh muscle needle biopsies from volunteers: We therefore performed a prospective evaluation of the rate of disappearance of GLUT3 mRNA and protein in human skeletal muscle obtained from an autopsy.

Other reports of little or no GLUT3 mRNA have included autopsy specimens from patients dying of cancer[7] or of unspecified diseases.[8] Our studies demonstrated that GLUT3 protein is rapidly immunologically undetectable in autopsy specimens. Even at 4°C, half of the GLUT3 protein is not recognizable by 2 hours. GLUT3 mRNA and other mRNAs are more slowly lost, with a T_{50} (incubation time required for RPA signal to decrease to 50% of the starting value) of 5 to 24 hours at 4°C. At room temperature (20°C), the mRNA T_{50} was 2.2 hours for GLUT3 and 0.8 to 2.1 hours for the other four mRNAs tested. The postmortem time course of the temperature decline in various parts of the body may be different, with more central trunk tissues (eg, liver or psoas muscle) being the last to achieve 4°C in the refrigerated area of the morgue. This could cause different tissues in the same cadaver to have dramatically different protein and mRNA recovery.

Finger et al[18] evaluated the stability of mRNA and protein in

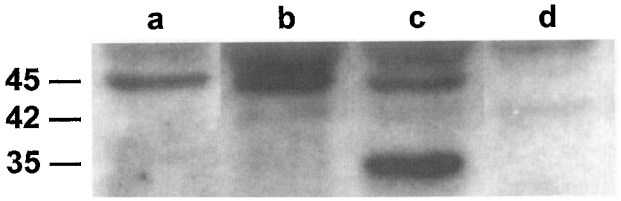

Fig 3. Immunoblots for GLUT3 protein in four muscle specimens from autopsies. The lanes contain 20 μg membrane protein from muscle specimens obtained 4 hours postmortem (a), 6 hours postmortem (b), 8 hours postmortem (c), and 18 hours postmortem (d). The apparent molecular weight (kd) for 3 specifically labeled bands is shown at left.

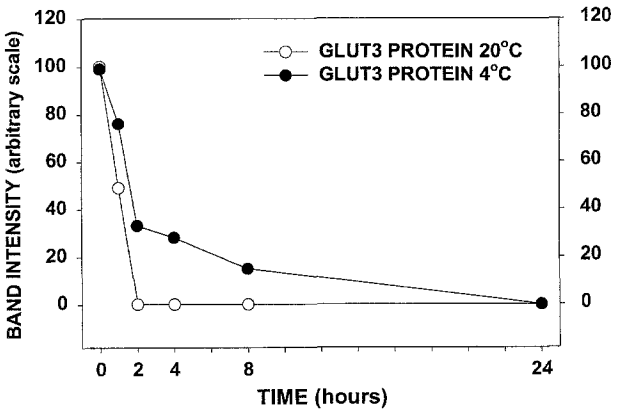

Fig 4. Time course of GLUT3 protein degradation as measured using an immunoblot. Data represent the analysis of 1 immunoblot using ImageQuant software to quantify a digitized ECL film. This experiment is typical of 3 separate immunoblots of incubations performed at the 2 temperatures indicated.

human autopsy liver slices incubated at either 37°C or 4°C for up to 16 hours. Two-dimensional electrophoresis of proteins could not identify significant differences between fresh and incubated liver soluble proteins. RNA integrity was evaluated by electrophoresis and comparison of the amount of RNA in the 28S band versus the 18S band. They found large declines in 28S RNA and erratic 28S:18S ratios, but there was no relationship to the duration of incubation. Their two starting liver specimens were obtained 35 and 45 minutes after cardiac death in trauma victims. They did not evaluate the immunological integrity of any protein. They concluded that protein and RNA in liver are stable enough to allow meaningful studies on tissue collected under standard autopsy conditions.[18] It is apparent from their data that substantial degradation occurred prior to the incubations and that the results were erratic enough that further degradation may have been obscured by technical variability in RNA isolation or the two-dimensional protein electrophoresis procedure. Larsen et al[19] evaluated tissue samples of seven organs from 10 autopsies for the utility of RNA in Northern blots or DNA in Southern blots. They concluded that RNA was usable up to 2 days and DNA up to 5 days for Northern and Southern blots.[19] Humphreys-Beher et al[20] used autopsy specimens in studies of cystic fibrosis. They isolated RNA from the lung, stomach, and pancreas for the purpose of generating cDNA libraries. These investigators found that biologically active mRNA was recovered from the lung up to 20 hours after death, but the stomach and pancreas must be harvested within 2 hours in a strong denaturant buffer to inactivate nucleases.[20]

The relative amount of mRNA and protein for GLUT1, GLUT3, and GLUT4 that is present in normal skeletal muscle may also bear on the signal intensity obtained in samples from autopsies. Recent studies from our laboratory have demonstrated that normal human muscle has threefold higher mRNA levels for GLUT4 versus GLUT3. mRNA concentrations were about twofold higher for GLUT1 compared with GLUT3. If these three had similar rates of degradation, then the GLUT3 signal would disappear first. We have not quantified the relative amount of the three glucose transporter proteins, but the same principle may apply to the loss of detectable protein. This would be further accentuated if GLUT3 protein is more rapidly degraded, as it appears to be, relative to GLUT4 protein.

Kleiner et al[21] recently reviewed the use of autopsy material for molecular pathology. Their overview of the problems with recovery of useful DNA, RNA, or proteins concluded that DNA was the most stable of the three materials often sought at necropsy in quantities not available from living subjects. DNA may be recoverable for several days after death, but it is fragmented by Formalin in fixed tissues.[21] RNA is less recoverable because of its inherent instability and because of the tissue content of degradative enzymes. Protein preservation is much more variable and depends heavily on the particular protein and the tissue from which it is sought.[21]

Muscle specimens obtained at autopsy are highly variable in the recovery of useful mRNA or specific protein, and specimens obtained more than 6 hours after death are unlikely to be useful. Autopsy specimens may dramatically underestimate the amounts of mRNA and protein that were present in the tissue prior to death. We conclude that the erroneous suggestion that GLUT3 expression is not present in adult human skeletal muscle is largely due to the rapid degradation of GLUT3 protein and mRNA in the autopsy specimens previously evaluated.

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