Glyceraldehyde-3-Phosphate Dehydrogenase Varies With Age in Glycolytic Muscles of Rats

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, protein, and enzyme activity levels in hindlimb muscles of adult and senescent Fischer 344 x Brown Norway rats were investigated. Soleus muscles from adult and senescent rats had similar levels of GAPDH. In contrast, muscles containing a large proportion of glycolytic fibers had lower GAPDH levels in senescent rats relative to these muscles in adult rats; this was observed at both the mRNA and protein levels. These data indicate that skeletal muscle glycolytic capacity of fast muscles is diminished with age and that it may be caused by changes at the level of transcription. Also, because GAPDH mRNA levels change with age in several rat muscles, GAPDH mRNA is not always a proper internal control for mRNA analyses of aging skeletal muscle.

METHODS

Animals

Fischer 344 x Brown Norway F1 hybrid male rats aged 9 months (adult, n = 6) and 37 months (senescent, n = 6) were studied. Thirty-seven months represents the age at which these animals attain ~50% mortality (8). Animals were housed in pathogen-free conditions, two per cage at 20–22°C with a 12-hour light/dark cycle, and received rat chow and water ad libitum. All animals appeared healthy, e.g., they were able to ambulate normally and food and water consumption was standard. Animals were then anesthetized with pentobarbital sodium (55 mg/kg intraperitoneally) and gastrocnemius, plantaris, soleus, and extensor digitorum longus (EDL) muscles were excised from the right hindlimbs. Animals were euthanized with an overdose of pentobarbital sodium. Plantaris, soleus, and EDL muscles were quickly cut into three pieces and immediately frozen in liquid nitrogen. The deep, red portion of the gastrocnemius muscle was separated from the whole muscle, cut into three pieces and quickly frozen. All tissues were stored at −80°C.

mRNA Analyses

GAPDH RNA probe was made from a reverse-transcribed–polymerase chain reaction (RT-PCR) product. Total RNA was extracted from rat gastrocnemius muscle and 5 μg was reverse-transcribed in a total volume of 20 μL (SuperScript II RNase H– Reverse Transcriptase, Life Technologies, Gaithersburg, MD). A 110-bp segment of GAPDH was amplified using 1 μL of cDNA, 50 ng of each primer (5'-AAATGGGGTGATGCTGGTG and 3’-GAGATGATGACCCTTTTGG), 250 μM dNTPs, 1× polymerase chain reaction buffer (Sigma Chemical Co, St. Louis, MO), and 2 units Taq DNA polymerase (Sigma Chemical Co, St. Louis, MO) using an annealing temperature of 54.2°C. The resulting GAPDH RT-PCR product was cloned using pCR-Script Amp SK(+)
Cloning Kit (Stratagene, La Jolla, CA). The insert was verified by DNA sequencing (Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida) and was >99% similar to the previously reported rat GAPDH sequence (9). RNA probes were made by in vitro transcription as described previously (10) using BrightStar BIOTINscript Kit (Ambion, Austin, TX). The resulting GAPDH probe had ~10% of the biotinylated nucleotides.

RNA probes were also generated to detect cyclophilin mRNA and 28S-rRNA (Rat Internal Standard Kit, Ambion). Transcriptions were done under the control of T7 polymerase and the resulting cyclophilin and 28S probes had 100% and ~2% of the biotinylated nucleotides, respectively. All RNA probes were gel-purified, extracted, solubilized in 20 μL of RNase-free H₂O and stored in 5 μL aliquots at ~80°C. Aliquots were diluted 100-fold prior to use.

For Northern blots, total RNA was extracted from muscles (~100 mg piece of each muscle) using 1 mL of TRI-Reagent (Molecular Research Center, Cincinnati, OH) per muscle and a mechanical homogenizer. Extracted RNA was solubilized in 20 μL of RNase-free H₂O, quantitated in duplicate by absorbance at 260 nm, and then stored at ~80°C. Total RNA was fractionated on 1% formaldehyde-agarose gels and transferred to nylon using a rapid downward transfer system (Turboblotter, Schleicher & Schuell, Keene, NH). Following transfer, membranes were stained with methyl blue (Molecular Research Center, Cincinnati, OH) for 10 seconds, rinsed several times in distilled H₂O and then dried on filter paper. RNA was immobilized by ultraviolet crosslinking and membranes were cut such that the upper part contained the 18S-rRNA bands and the lower part contained the 28S-rRNA bands. Membranes were prehybridized for 3 hours at 60°C in 5× Denhardt’s solution, 5× sodium chloride/sodium citrate buffer (SSC), 1% sodium dodecyl sulfate (SDS), 50% formamide, and 0.1 mg/mL tRNA. Hybridization was done overnight at 60°C in fresh prehybridization buffer containing 10% dextran sulfate and RNA probe (10 μL GAPDH or cyclophilin RNA probe/ml buffer for lower membranes and 10 μL 28S RNA probe/ml buffer for upper membranes). Following hybridization, membranes were washed at room temperature twice in 2× SSC and 0.1% SDS, twice in 2× SSC and 0.25% SDS, and then once at 42°C in 2× SSC and 0.25% SDS. Signals were detected on film by chemiluminescence (BrightStar BioDetect Nonisotopic Detection, Ambion) and then quantitated (Bio Image, Genomic Solutions, Ann Arbor, MI). GAPDH bands were located just beneath the 18S-rRNA band at ~1.4-kb and cyclophilin bands were located at ~0.7 kb.

Western Blotting

Muscle GAPDH protein levels were determined by Western blotting. A 20–30-mg piece of muscle was homogenized in 19 volumes of 10 mM sodium phosphate buffer (pH 7.6) on ice. Insoluble material was pelleted by centrifugation at 5000 g for 10 minutes at 4°C and the supernatant was freeze-thawed. Protein concentration of the supernatant was determined spectrophotometrically using BCA Protein Assay and bovine serum albumin standards (Pierce, Rockford, IL). Twenty-five micrograms of protein from each sample was separated on 12% acrylamide sodium dodecyl sulfate–polyacrylamide gels. Proteins were blotted to nitrocellulose and probed with anti–glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (Chemicon International, Temecula, CA) diluted 1:1000 in I-Block (Tropix, Bedford, MA) for 30 minutes at 25°C. Single bands corresponding to a molecular mass of ~37 kDa were detected by chemiluminescence (CDP-Star, Tropix). These bands were quantitated using Bio Image (Genomic Solutions).

Enzyme Activity

GAPDH activity in muscles was determined at 25°C by measuring NADH oxidation (11). The assay mixture contained 0.2 mM β-NADH, 6 mM glyceraldehyde-3-phosphate, 0.9 mM EDTA, and 1.7 mM MgSO₄ in 82.5 mM triethanolamine buffer (pH 7.6). Muscle sample (8 μL of supernatant used in Western blots) was added to the assay mixture, and the reaction was started by the addition of 1.1 mM ATP and 14.8 U/mL phosphoglyceric phosphokinase. The total assay volume was 0.5 mL. Absorbance at 340 nm was read every 5 seconds for 1 minute. Each sample was assayed in triplicate.

Statistics

Two-way analysis of variance tests with Student-Newman-Keuls post hoc tests were used to determine differences in mRNA, protein, and enzyme activity levels. Student’s t tests were used to determine if differences in 28S and cyclophilin RNA levels existed between muscles from adult and senescent rats. An α level of .05 was used for all tests.

Results

RNA Levels

GAPDH mRNA levels were significantly lower in red gastrocnemius, plantaris, and EDL muscles from senescent rats compared to corresponding muscles from adult rats (main effect of age, p < .001; Figures 1A and 2). GAPDH mRNA levels were also different between muscles (main effect of muscle type, p = .030) and differences between age groups were dependent upon muscle type (interaction effect of age and muscle type, p = .027).

28S-rRNA levels did not differ between adult and senescent rats in any of the muscles studied (all ps ≥ .69; Figure 1B). 28S signals were therefore used to confirm that samples were loaded onto gels and transferred to membranes equally (each 28S signal did not vary from the mean of the other 28S signals on that membrane by more than 8%). Cyclophilin mRNA levels in plantaris and soleus muscles did not differ between adult and senescent rats (all ps ≥ .42; Figure 3).

Protein Levels

GAPDH protein expression in plantaris and EDL muscles from senescent rats was ~50% of that in adult muscles, whereas those in red gastrocnemius and soleus muscles did not differ between adult and senescent rats (main effect of age, p < .001; Figures 4 and 5). GAPDH protein levels
were different between muscles (main effect of muscle type, \( p < .001 \)) and age-related differences in GAPDH protein levels were dependent upon the muscle type (interaction between age and muscle type, \( p = .032 \)).

**Enzyme Activity**

GAPDH enzyme activities in red gastrocnemius, plantaris, and EDL muscles from senescent rats were 30–50% lower than those in muscles from adult rats (main effect of age, \( p < .001 \); Figure 6). Differences in enzyme activities were found between muscles (main effect of muscle type, \( p < .001 \)), and age-related differences in GAPDH activities were dependent upon the muscle type (interaction between age and muscle type, \( p < .001 \)). Enzyme activity data normalized by protein content (instead of by wet weight) yielded the same results. However, when normalized to the GAPDH protein levels in the corresponding muscles, GAPDH activities were not different between muscles from adult and senescent rats (main effect of age, \( p = .28 \)).

**Discussion**

Changes in skeletal muscle during aging result in a decline in muscle function and use. Based on studies of human muscle, however, the glycolytic capacity of skeletal muscle

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**Figure 1.** Representative Northern blots of GAPDH mRNA (A) and 28S rRNA (B) in skeletal muscles from 9 months old (Adult) and 37 months old (Senescent) rats. Eight microgram of total RNA was loaded per lane.

**Figure 2.** GAPDH mRNA expression in muscles from adult and senescent rats. Values from adult animals were set to 1.0. Values are means ± SE (n = 6). Gastroc, red gastrocnemius muscle; EDL, extensor digitorum longus muscle. *Significantly different from corresponding adult.

**Figure 3.** Representative Northern blots of cyclophilin mRNA. Twenty-five microliter of total RNA was loaded per lane.

**Figure 4.** Representative Western blots showing GAPDH protein detection by anti-glyceraldehyde-3-phosphate dehydrogenase antibody. Twenty-five microgram of protein was separated from muscles of 9 months old (Adult) and 37 months old (Senescent) rats. The molecular weight markers represent 45 and 29 kDa.
is one property that has been suggested to be unaffected by age (12). Data from rat muscle, on the other hand, indicate that the glycolytic capacity may diminish with age (1–3).

The present findings of GAPDH support the contention that the carbon flux through the glycolytic pathway is diminished in some muscles of aged animals. Furthermore, from the present study it can be deduced that the lower glycolytic capacity of muscles from aged animals may be related to reduced transcription because GAPDH mRNA levels were lower in many muscles of the senescent rats. We cannot speculate on the pattern of decline; for example, we do not know if glycolytic capacity is gradually reduced though out the entire aging process or if there is some critical age beyond which the reduction occurs. Additional studies are required to determine these mechanisms.

It is likely that many of the observed differences in GAPDH between different muscles and between muscles from animals of different ages are related to the fiber-type compositions and age-related changes in fiber-type composition, respectively. In general, there is a transition to slower, more oxidative fibers with aging (13). Soleus muscle of adult rats is composed of ~90% slow-twitch oxidative (SO) fibers and ~10% fast-twitch oxidative glycolytic (FOG) fibers (14). With age there is a slight increase to an even greater percentage of SO fibers in this muscle (13).

GAPDH protein level and enzyme activity in soleus muscle was the lowest of any of the muscles studied and did not differ between adult and senescent rats. This indicates that glycolytic capacity does not change with age in oxidative muscles. In contrast, red gastrocnemius, plantaris, and EDL muscles of adult rats have mixed fiber-type compositions (1–35% SO fibers, 20–56% FOG fibers, and 9–79% FG fibers) (14). With age the fiber-type compositions of these muscles shift to more SO and FOG fibers and less FG fibers (13). In general, we found that GAPDH protein level and enzyme activity in those muscles were lower in senescent rats, suggesting that glycolytic capacity is diminished with age in muscles containing a substantial number of glycolytic fibers.

Age-related changes in enzyme molecules have been well documented and have been attributed to a wide variety of post-translational modifications (15). One specific mechanism of the age-related decrease in GAPDH enzyme activity has been shown to lie in the four NAD$^+$ binding sites of the enzyme (16). Conformational changes occur in the NAD$^+$ binding domains of GAPDH enzyme from muscle of older animals, which result in higher dissociation constants and ultimately reduced enzymatic activity (16). From those findings it was concluded that age-related decreases in GAPDH activity occur solely through post-translational processes. In contrast, our data show that when GAPDH activity is normalized to GAPDH protein levels in the muscles, enzyme activity per se is not affected by age. Therefore, our findings suggest that the decreased enzyme activity is the result of transcriptional modification, and possibly pretranslational and translational modifications as well, especially in muscles containing glycolytic fibers.

In the present study we provide evidence that GAPDH mRNA, a commonly used internal control in mRNA analyses of muscle, is reduced with age in several rat muscles. Internal controls in methods such as Northern blotting, ribonuclease protection assays, and RT-PCR are necessary in order to account for sample to sample variations that occur, for example, during RNA isolation, loading, transferring and blotting, or amplification. An internal control is valid only if it is not affected by the experimental treatment, e.g., GAPDH mRNA levels in muscles must not change as a function of age. Only when this condition is met can it be assumed that variations observed in the internal controls are the result of experimental errors and not due to the experimental treatment. Our data indicate that GAPDH mRNA is not a valid internal control for many experiments designed to analyze mRNA in aging rat muscle.

GAPDH mRNA levels may change with age differently in other experimental animals. Musaro and coworkers (6) did not report a difference in GAPDH mRNA levels in aging mouse muscles. However, they apparently pooled several mouse hindlimb muscles for the mRNA experiments, but statistical analyses to verify that GAPDH mRNA levels did not differ across age groups were not reported. Muscle GAPDH mRNA levels may also change as the result of experimental manipulations. It has been shown that denervation (17), chronic stimulation (18), and overload-induced hypertrophy (19) cause GAPDH mRNA levels in skeletal muscles to decline, whereas hindlimb suspension results in...
increases (20). However, the occurrence of these changes may depend upon the muscle being studied. For example, Mozdziaik and colleagues (7) reported no change in GAPDH mRNA levels in overloaded rat soleus muscle whereas Tsika and colleagues (19) reported a 200% decrease in GAPDH mRNA levels in overloaded rat plantaris muscle.

β-Actin, α-tubulin, and the rRNAs 18S and 28S are other “housekeeping” genes whose transcripts are frequently used as internal controls in mRNA analyses of human, mouse, and rat tissues (Ambion and Clontech of Palo Alto, CA, have probes/primers for these genes). Using Northern blot analyses we have found that levels of 18S and 28S rRNAs do not differ in muscles of adult and senescent rats. However, because of the abundance of these rRNAs they are not optimal internal controls for many experiments. An alternative internal control for mRNA analyses in studies of aging muscle that we have found to be satisfactory is cyclophilin. Cyclophilin is a constitutively expressed gene that encodes a cytosolic protein essential for the correct folding of several proteins. We have analyzed cyclophilin mRNA by Northern blotting, as well as by ribonuclease protection assay and RT-PCR (unpublished results), and found that levels are constant in soleus and plantaris muscles of rats from 4 to 37 months of age. In summary, internal controls for mRNA analyses in skeletal muscles should be selected based upon the experimental manipulations used; cyclophilin may be a good alternative in lieu of the traditional GAPDH.

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