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Single muscle fiber gene expression in human skeletal muscle: validation of internal control with exercise

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Abstract

Reverse transcription and real-time PCR have become the method of choice for the detection of low-abundance mRNA transcripts obtained from small human muscle biopsy samples. GAPDH, β-actin, β-2M, and 18S rRNA are widely employed as endogenous control genes, with the assumption that their expression is unregulated and constant for given experimental conditions. The aim of this study was to determine if mRNA transcripts could be performed on isolated human single muscle fibers and to determine reliable housekeeping genes (HKGs) using quantitative gene expression protocols at rest and in response to an acute exercise bout. Muscle biopsies were obtained from the gastrocnemius of three adult males before, immediately after, and 4 h following 30 min of treadmill running at 70% of VO_2max. A total of 40 single fibers (MHC I and IIa) were examined for GAPDH, β-actin, β-2M, and 18S rRNA using quantitative RT-PCR and SYBR Green detection. All analyzed single fiber segments showed ribosomal RNA (28S/18S). No degradation or additional bands below ribosomal were detected (rRNA ratio 1.5–1.8). Also, no high or low-molecular weight genomic DNA contamination was observed. For each housekeeping gene the duplicate average SD was ±0.13 with a CV of 0.58%. Stable expression of GAPDH was observed at all time points for each fiber type (MHC I and IIa). Inconsistent expression of β-actin, β-2M, and 18S rRNA was observed during the post-exercise time points for each fiber type. These data indicate that successful extraction of high quality RNA from human single muscle fibers along with quantification of mRNA of selected genes can be performed. Furthermore, exercise does influence the expression of certain HKGs with GAPDH being the most stable.

Keywords: Endogenous genes; RT-PCR; Muscle fiber; Housekeeping genes; SYBR Green; Running

Recently, our laboratory has been engaged in human exercise studies that have isolated individual slow- (MHC I) and fast (MHC IIa)-twitch single muscle fibers to determine their functional and structural properties [1–4]. These data show that single muscle fibers respond differently to a variety of external stimuli (exercise, disuse, etc.). Furthermore, the type of alterations that we have observed varies among the MHC I and IIa muscle fiber types. To better understand the various developmental and metabolic conditions that persist in individual human skeletal muscle fiber types, it would be informative to characterize selected functional and regulatory genes and how they are altered with exercise.

A valuable tool for the accurate quantification of gene expression is fluorescent quantitative real-time RT-PCR. This technique demonstrates a high degree of sensitivity when applied to the expression of selected genes from limited tissue samples such as those obtained from human muscle biopsy samples. Real-time RT-PCR also allows for highly accurate template quantification from a minimal amount of tissue [5–7]. Thus, by applying the high throughput capacity of real-time PCR to single muscle fiber segments obtained from human muscle biopsy samples it will be possible to expand the functional and structural information to include specific aspects of molecular biology in the adaptation to exercise. This is a desirable goal given that human skeletal muscle is composed of a continuum of muscle fiber types with a wide range of functional characteristics [8].

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The relative RT-PCR quantifies the abundance of the gene of interest (GOI) against an endogenous control, such as housekeeping genes (HKGs) that exhibit stability with a high degree of expression in various types of tissues. The use of quantitative gene expression protocols requires adequate internal controls to monitor intersample variation. Therefore, the unregulated and constant nature of HKGs needs to be established for a given experimental condition and type of tissue [9].

Commonly used HKGs in studies measuring mRNA expression in the genes of interest are: 18S and 28S ribosomal RNA (18S and 28S); β-actin; glyceraldehyde-3-phosphate dehydrogenase (GAPDH); and β-2-microglobulin (β-2M). Recently, several papers have been published looking at the viability of using certain genes as HKGs and it is clear that there is no single HKG that is suitable for all experimental conditions [9–12]. Despite the common use of above-listed HKG templates, no comprehensive analysis has been performed to confirm their stable expression in exercise-induced MHC I and MHC IIa single muscle fibers.

In an attempt to validate the four common HKGs used as an endogenous control for analysis of gene expression in human single muscle fibers, we hypothesized the following: (1) total RNA integrity would be adequate for subsequent RT-PCR analysis using a novel RNase inhibitor reagent that allows efficient preservation of tissue/fibers during time-consuming microdissection of single fiber, (2) using SYBR Green detection, reliable real-time PCR could be performed from a minute amount of starting material (human single muscle fiber segment, ~4 mm in length), and (3) GAPDH, β-actin, 18S rRNA, and β2M expression could be quantified in slow- (MHC I) and fast-twitch (MHC IIa) muscle fibers to determine their stability before and after aerobic exercise.

Materials and methods

Subjects

Three healthy men between 20 and 30 years of age were recruited for the study. They were non-obese (less than 28 kg/m^2), normotensive, and non-smokers. Prior to giving their consent to participate in this study, these individuals were fully informed of the risks and stresses associated with the run exercise and muscle biopsies used in the research. Informed consent was obtained from each volunteer. The Human Research Committee of Ball State University approved the experimental protocol.

Run exercise

Maximal oxygen uptake. Subjects performed a continuous incremental treadmill run to volitional exhaustion to determine VO_2max. Throughout these stages the subjects were asked to rate their perception of effort using a Borg Scale [13]. When the subject's rating was greater than 13 for a given speed, the grade on the treadmill was subsequently increased by 2% every 2 min until exhaustion. Maximal oxygen uptake (VO_2max) was confirmed by a plateau of VO_2 and a respiratory exchange ratio (RER) greater than 1.10 with an increase in the incline of the treadmill.

During the test, expired air was measured at 30-s intervals with an automated open-circuit system that incorporated a Parkinson-Cowen dry-gas meter, 3 L mixing chamber, and electronic O_2 (Applied Electrochemistry-Ametek S-3A) and CO_2 (Applied Electrochemistry-Ametek CD-3A) analyzers which were interfaced to an IBM computer. The gas analyzers were calibrated prior to each test with standardized gases that had previously been analyzed by the Haldane method.

Submaximal test run. Approximately 1 week after the VO_2max test, each subject completed the submaximal test run. Each subject's VO_2max was used to determine the selected treadmill pace that corresponded to ~70% effort. Following an adequate warm-up of stretching and walking on the treadmill, subjects ran on a treadmill for 30 min at approximately 70% of their VO_2max. Relative intensity was confirmed at 15 and 30 min of the run by collecting expired air samples into Douglas bags. The expired air samples were measured using the same open-circuit system described above.

Muscle biopsy

Muscle biopsies [14] were obtained from the lateral aspect of the gastrocnemius (calf) muscle from each subject before, immediately after, and 4 h post-exercise. Each muscle sample was immediately divided, placed in 0.5 ml RNA *later* solution (Ambion, Austin, TX), and stored at −20 °C until analysis.

Muscle fiber separation and RNA extraction

Individual muscle fiber segments (~6 mm in length) was isolated under a light microscope. A small bundle of muscle fibers was placed in a petri dish filled with RNA later. Each myofiber was dissected from the myofiber bundle using fine tweezers. All isolated fibers were intact, unbroken and of the full length of the bundle. The individual fibers were cleaned of connecting tissue and any artifact. The fibers were checked under an inverted microscope to observe the potential presence of satellite cells. Because some satellite cells are contoured to fibers and often stay attached to the external laminae of the fiber, it was not possible to completely remove all satellite cells without a digestion process. In this study, we isolated single muscle fibers from a small bundle without digestion and satellite cell separation. Taking into consideration that previous research has shown satellite cells in muscle are mitotically quiescent and transcriptionally less active than myonuclei [15,16], we feel the current isolation technique is adequate for real-time RT-PCR application. Furthermore, research that applies a muscle homogenate approach for gene expression studies would not be able to remove any potential artifacts within the interior of the muscle sample to be used for analysis. Thus, the single muscle fiber approach represents the opportunity to study fiber-specific gene expression with minimal to no contribution from satellite cell gene expression.

Following the isolation of each muscle fiber segment, one-third of the muscle fiber was clipped and placed in SDS sample buffer (for fiber type identification by SDS–PAGE; described below). The remaining part of the muscle fiber (~3–4 mm) was placed into a tube with 0.8 ml of the RNA extraction reagent and 6 μl polyacryl carrier (TRI Reagent; Molecular Research Center, Cincinnati, OH). Extracted RNA isolation for each individual muscle fiber was completed according to the manufacturer's protocol. Total RNA was dissolved in 12 μl of 0.1 mM EDTA. The total RNA from a single muscle fiber is relatively low in comparison to total RNA from a small (10–15 mg) muscle tissue sample. Given that DNase treatment of nuclei acid extracts from small tissue specimens has been shown to degrade or destroy RNA [17–19], DNase treatment was not applied to the single muscle fibers in this study.

RNA quality control by microfluid electrophoresis

The 1 μl of single fiber total RNA solution (1:3 v/v) was analyzed using the RNA 6000 Pico LabChip [20] on a 2100 Bioanalyzer (Aligent Technology, Palo Alto, CA). This system reported detailed information about the condition of RNA samples (integrity and purity). The sample was eletcrophoretically separated into two peaks of 18S and 28S ribosomal RNA. Data were displayed as a gel-like image and/or an electropherogram. Sample analyses were performed as described by the manufacturer. The high quality of RNA was confirmed by presence of ribosomal peaks with no additional signals (DNA contamination or RNA degeneration) below the ribosomal bands and no shift to lower fragments.

Reverse transcription

Reverse transcription (RT) was done using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). This system has been optimized for sensitive RT-PCR on low input of RNA and is designed to generate first-strand cDNA from total or poly(A)$^+$RNA. Total RNA amounts were not normalized between samples. RT was performed in two steps in a total reaction volume of 25 μl. The first reaction mix contained: 10 μl of total RNA extract, 1.25 μl oligo(dT)$_{12–18}$ (0.5 μg/μl), 1.25 μl dNTP mix (10 mM), and 0.25 μl DNase- and RNase-free water. The mixture was incubated at 65 °C for 5 min, chilled on ice for 1 min, and added to the reaction mixture of the second step which contained: 2.5 μl of 10× RT buffer, 5.0 μl of 25 nM $MgCl_2$, 2.5 μl of 0.1 M DDT, and 1.0 μl RNaseOUT recombinant RNase inhibitor. The final mixture was incubated at 42 °C for 2 min. Following incubation, 1 μl of SuperScript II RT (50 U) was added to each tube and incubated for 50 min, followed by reaction termination at 70 °C for 15 min and chilled on ice. All incubations were done using a DNA Engine, Peltier thermal cycler (MJ Research, Waltham, MA). The resulting cDNA samples were diluted to a final volume of 60 μl (1:6). The remaining 2 μl of total RNA extract was diluted to a final volume of 12 μl (1:6) and used to run non-RT PCR control.

Real-time PCR SYBR Green detection

Quantitative PCR was performed using a Rotor-GeneTH 3000 centrifugal real-time cycler (Corbett Research, Mortlake, NSW, Australia) using SYBR Green Real-time PCR Buffer (BioSource, Camavillo, CA). Each reaction contained: 2.5 μl of the 10× SYBR Green PCR Buffer, 0.625 μl dNTPs (10 mM), 0.4 μl Platinum Tag DNA polymerase (1 U), 2.5 μl of 10× Primer Pairs in case of GAPDH, β-actin, and 18S rRNA (BioSource, Camavillo, CA) and 1 μl of forward and reverse primers each in case of for $\tilde{\beta}$2M (10 mM) (Invitrogene, Carlsbad, CA), 2.5 μl cDNA (1:6 RNA dilution), and water to a final volume of 25 μl. Amplification was performed in 0.1 ml real-time PCR tubes (Corbett Research, Mortlake, NSW, Australia) placed in the 72-well rotor of the Rotor-Gene instrument. PCR parameters were as follows: initial denaturation at 95 °C for 2 min to activate Platinum Tag DNA polymerase followed by 45 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C (with fluorescence data acquired, gain set at 8 for SYBR Green). Following the final cycle, melting curve analysis was performed to examine the specificity in each reaction tube (absence of primer dimers and other nonspecific products). The Rotor-Gene software allows automatic melting curve analysis for all tested samples in a given run. SYBR Green fluorescence of the generated products was continuously monitored throughout the temperature ramp from 60 to 99 °C. The temperature rose in 1 ° increments with a 5-s hold at each degree. A single melt peak for each reaction confirmed the identity of each PCR product.

Human housekeeping GAPDH, β-actin, 18S rRNA, and β-2M served as reference and target genes to each other in assessing the overall cDNA content in the tested samples. Human β-2M was custom-made by Invitrogene (Carlsbad, CA) using the oligo sequence published in RTPrimerDB; ID 2 (Jo Vandesompele, Gent University, Belgium). All primers used in this study were designed for gene expression analysis and were "mRNA-specific."

Real-time PCR data analysis

The influence of running exercise on muscle fiber HKG expression was evaluated by a relative quantification method. The method is based on the fact that the difference in threshold cycles (ΔC_t) between the gene of interest (GOI) and HKGs is proportional to the relative expression level of the GOI. To ensure the accuracy of the obtained data, the reaction efficiencies of the two assays should be similar or a correction factor must be introduced into the calculation (see the section Validation of the $2^{-\Delta\Delta C_t}$ methods).

For each amount of RNA tested, duplicate C_t values were obtained and averaged. The threshold for all PCR runs was set up at 0.01253 fluorescence units. The fold change in one tested HKG (target gene) immediately after (Post 0) and 4 h post (Post 4) exercise was normalized to other HKG (reference gene) and compared to the pre-exercise condition (Pre) using the following formula:

$$\text{fold change} = 2^{-\Delta\Delta C_t},$$

where $\Delta\Delta C_t = (C_{t\,\text{target}} - C_{t\,\text{reference}})_{\text{time}\,x} - (C_{t\,\text{target}} - C_{t\,\text{reference}})_{\text{time}\,0}$. time x is any time point for the target gene normalized to the reference gene and time 0 represents the 1x expression of the target gene normalized to the reference gene at time 0 (Pre). The fold change in the *one target housekeeping gene*, normalized to the *other housekeeping gene*, served as the reference gene, and relative to the expression at time zero (Pre), was calculated for each sample. Using this analysis, if the level of all four HKGs was not affected by experimental conditions the values of the mean fold change at each time point should be very close to 1 (i.e., since $2^0 = 1$) [21].

Validation of the $2^{-\Delta\Delta C_t}$ method

Serial dilutions (1; 0.5; 0.250; 0.125; 0.062; and 0.031) of positive control DNA for GAPDH, β-actin, and 18S rRNA (BioSource; Camarillo, CA) were amplified by real-time PCR using gene-specific primers. In the case of β-2M gene, the cDNA synthesized from the whole muscle tissue sample was used for serial dilutions and amplified with gene-specific primers. The ΔC_t ($\Delta C_{t\,\text{target}} - \Delta C_{t\,\text{reference}}$) for all six combinations among the HKGs was calculated for each dilution. A plot of the log cDNA dilution versus the ΔC_t was made. The slope was calculated along with the validation of the amplification efficiencies of the target and reference genes.

SDS–PAGE: MHC isoform identification

SDS–PAGE analysis was used to determine the myosin heavy chain (MHC) isoform (MHC I and MHC IIa) for each single muscle fiber. One-third of each muscle fiber was solubilized in 40 μl of 1% SDS sample buffer (1% SDS, 6 mg/ml EDTA, 0.06 M Tris, pH 6.8, 2 mg/ml bromphenol blue, 15% glycerol, and 5% β-mercaptoethanol). SDS–PAGE methods and procedure are described in detail elsewhere [22]. The MHC isoforms were identified according to their migration rates and compared to molecular weight standards for each specific fiber.

Statistic analysis

Data are presented as means ± SE. Before the statistical analysis was performed, all $2^{-\Delta\Delta C_t}$ values were logarithmically transformed to obtain normal distributed data. Changes in mRNA expression immediately and 4 h after exercise were tested using a one-way ANOVA followed by a post hoc Tukey test for multiple comparisons with unequal sample sizes. Significance was set at the $P < 0.05$ level.

Results

Single muscle fibers from the gastrocnemius were isolated at three different time points (Pre, Post 0, and Post 4). The MHC distribution of the isolated muscle fibers was 67% MHC I and 33% MHC IIa. Real-time PCR analysis was performed on 40 fibers. The average length of the fiber segment and number of fibers analyzed for each MHC isoform are presented in Table 1.

RNA degradation is typically studied by the analysis of the ribosomal RNA signals on a 1.5% agarose gel, but the amount of the total RNA from a single muscle fiber was too small to be visible on a routine gel. To determine the quality of RNA in the studied RNA extract, a microfluid system (Bioanalyzer 2100) was employed. All analyzed single fiber samples showed ribosomal RNA (28S/18S). No signals of degradation in the form of additional peaks (on the electropherogram) or additional bands below ribosomal (on the gel-like image) were detected (Fig. 1). Also, no high or low molecular weight genomic DNA contamination was detected (see Fig. 1; DNA contamination forms a distinctive hump on the beginning of the electropherogram, broad bands on gel or clogs the gel when contamination is 20% or higher).

Table 1
The average length and the number of the muscle fibers analyzed at the different experimental condition

	Length of the analyzed fiber segment	Number of fiber		
		Pre	Post 0	Post 4
MHC I	3.41 ± 0.49	9	8	10
MHC IIa	3.12 ± 0.39	4	5	4

$F = 1.08$; $p > 0.05$.

Housekeeping gene expression in each single muscle fiber was analyzed by real-time PCR and relative quantification. We have made the assumption that the selected HKGs do not vary in the expression level among the samples when normalized to each other. The use of the relative quantification method requires that the PCR amplification efficiencies of all genes be similar and preferably at or above 90%. The real-time PCR characteristics are presented in Table 2. All four genes had the same amplification rate and reaction efficiency. The results of the validation of $2^{-\Delta\Delta C_t}$ method for real-time quantitative PCR are presented in Table 3. Analysis of serial dilutions had a slope value of log input cDNA amount versus ΔC_t of 0.1 or less, indicating that efficiencies of all reactions were equal.

Figs. 2A and B illustrate a typical plot of data generated by real-time RT-PCR using SYBR Green detection. The PCR signal was initially below the detection limit (threshold) and increased with cycle number to cross a threshold. To verify that SYBR Green dye detected only one PCR product, for a specific gene, the samples were subjected to the heat dissociation protocol following the final PCR cycle. Fig. 2C illustrates a typical melting curve for GAPDH gene PCR products with only a single dissociation peak at 88.9 °C. Dissociation of the PCR constantly produced single peaks for β-actin (91.1 °C); β-2M (87.1 °C); and 18S rRNA (92.1 °C), demonstrating the presence of only one product of PCR.

Amplification of cDNA (2.5 ml) synthesized from 10 μl of volume of the total RNA extracted from the MHC I fibers revealed that only GAPDH expression normalized either to β-actin, 18S rRNA, or β-2M had not been significantly changed at 0 and 4 h after exercise when compared to pre-exercise condition (Fig. 3). However, when 18S rRNA, β-actin, or β-2M

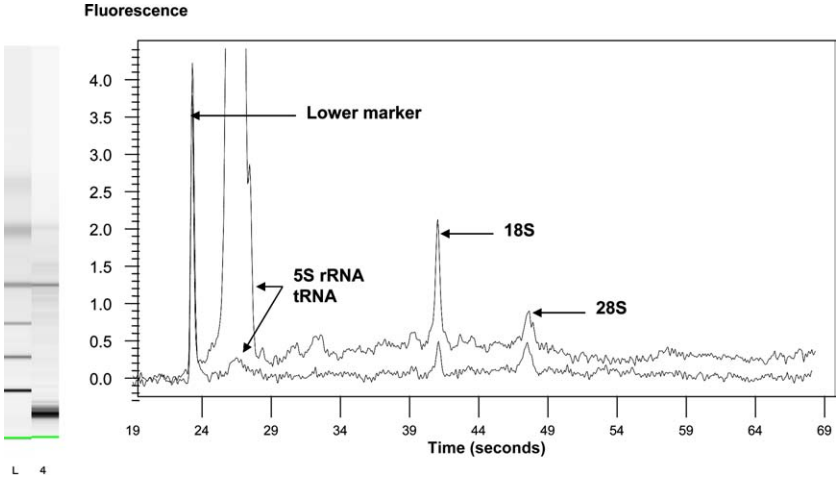

Fig. 1. The electropherograms of total RNA from human muscle analyzed using RNA 6000 Pico LapKit and 2100 Bioanalyzer. The total RNA from 5 mg muscle tissue (1:600 v/v) (lower line) and total RNA from single fiber (1:3 v/v) (upper line). The electropherograms and gel-like (L, 6000Ladder; 6, total RNA from single fiber) image show clear separation of total RNA to 18S and 28S subunits and on degradation products and no DNA contamination.

Table 2
Real-time PCR technique characteristics

	Genes			
	GAPDH	18S rRNA	β-Actin	β-2M
SD between duplicates	0.06	0.11	0.26	0.09
% CV per PCR run	0.36	0.51	1.01	0.43
Amplification rate	1.90	1.90	1.90	1.90
Efficiency	0.90	0.90	0.90	0.90
Threshold set/fluorescent units	12.53^{-2}	12.53^{-2}	12.53^{-2}	12.53^{-2}

Table 3
Test of amplicon efficiency done by plotting the log of the input template versus the ΔC_t; a slope of approximately zero (<0.1) demonstrates that efficiencies were equal

Target gene/reference gene	Dilution curve slope values
β-Actin/GAPDH	0.001
β-2M/GAPDH	0.103
18S rRNA/GAPDH	0.097
β-Actin/18S rRNA	0.055
β-2M/18S rRNA	0.016
β-2M/β-actin	0.036

was normalized to each other they showed marked changes in the expression in the post-exercise condition when compared to the pre-exercise condition. β-2M normalized either to β-actin or 18S rRNA showed an increased transcript level of 3- to 7-fold, respectively, at the Post 4 time point. 18S rRNA normalized to β-actin exhibited increased expression level throughout recovery time from 4-fold at Post 0 to 5-fold at Post 4 (Fig. 3).

Expression of the selected housekeeping genes for the MHC IIa fibers, when normalized to each other, showed a pattern of stability only for GAPDH and β-2M when normalized to β-actin (Fig. 4). GAPDH when normalized to 18S rRNA or β-2M showed variation in the transcript level at Post 4 from a decrease (90%) when normalized to 18S rRNA to an increase (9-fold) when compared to β-2M. 18S rRNA and β-2M had the largest change in expression at Post 4. 18S rRNA increased 20-fold when normalized to β-actin and β-2M increased 52-fold when normalized to 18S rRNA in comparison to pre- and immediately post-exercise.

Discussion

The current investigation presents relevant steps required to obtain total RNA and cDNA from individual human muscle fibers for gene expression studies using a commercially available RNase inhibitor and extraction kit. We were able to perform successful extractions of high quality total RNA from different muscle fiber segments which resulted in reproducible quantification of mRNA of HKGs using real-time PCR and the SYBR green detection system. From these data we demonstrate, for the first time, that the use of real-time PCR allows for a systematic evaluation of mRNA expression from starting material as little as one isolated muscle fiber. We also show that run exercise does influence the expression of certain HKG indicating that the internal control genes need to be carefully monitored and selected based upon treatment conditions.

This is the first study to report that run exercise influences the expression of selected housekeeping genes and that the expression of these genes is regulated differently between muscle fiber types. Running stimulated an increase in expression of β-actin, β-2M, and 18S rRNA to a greater extent at 4 h after exercise than immediately after exercise in both tested muscle fiber types. As a result, the housekeeping genes of β-actin, β-2M, and 18S rRNA, when normalized to each other, do not appear to be stable in response to exercise. In contrast, GAPDH was relatively stable in response to the exercise bout, thereby supporting the use of GAPDH as an internal control gene for quantitative expression studies of exercise-stimulated MHC I and MHC IIa muscle fibers. Recent analysis of internal standards that are typically used, like GAPDH, β-2M, 18S rRNA or 28S rRNA, confirmed their usefulness as appropriate internal standards in their quantification system [23–25]. While these internal control housekeeping genes may be appropriate for certain biological systems, our data indicate that traditional internal control genes can fluctuate with exercise and should be carefully monitored and evaluated in human muscle studies that use exercise as an intervention.

Ideally, the conditions of the experiment should not influence the expression of the internal control/housekeeping genes. However, it is unlikely that such a gene would exist since biological systems are dynamic and constantly responding to their environment [26–29]. Therefore, choosing a valid internal control for monitoring intersample variation should be mandatory. The most appropriate internal control gene would be one that has the least variation in its expression under experimental conditions.

The concept of employing real-time PCR techniques to tissues of small mRNA quantity is not new. Manual microdissection techniques have isolated single cells from renal biopsies [6,30] or single-cell cDNA precipitation protocols [7], which proved to be successful in quantifying

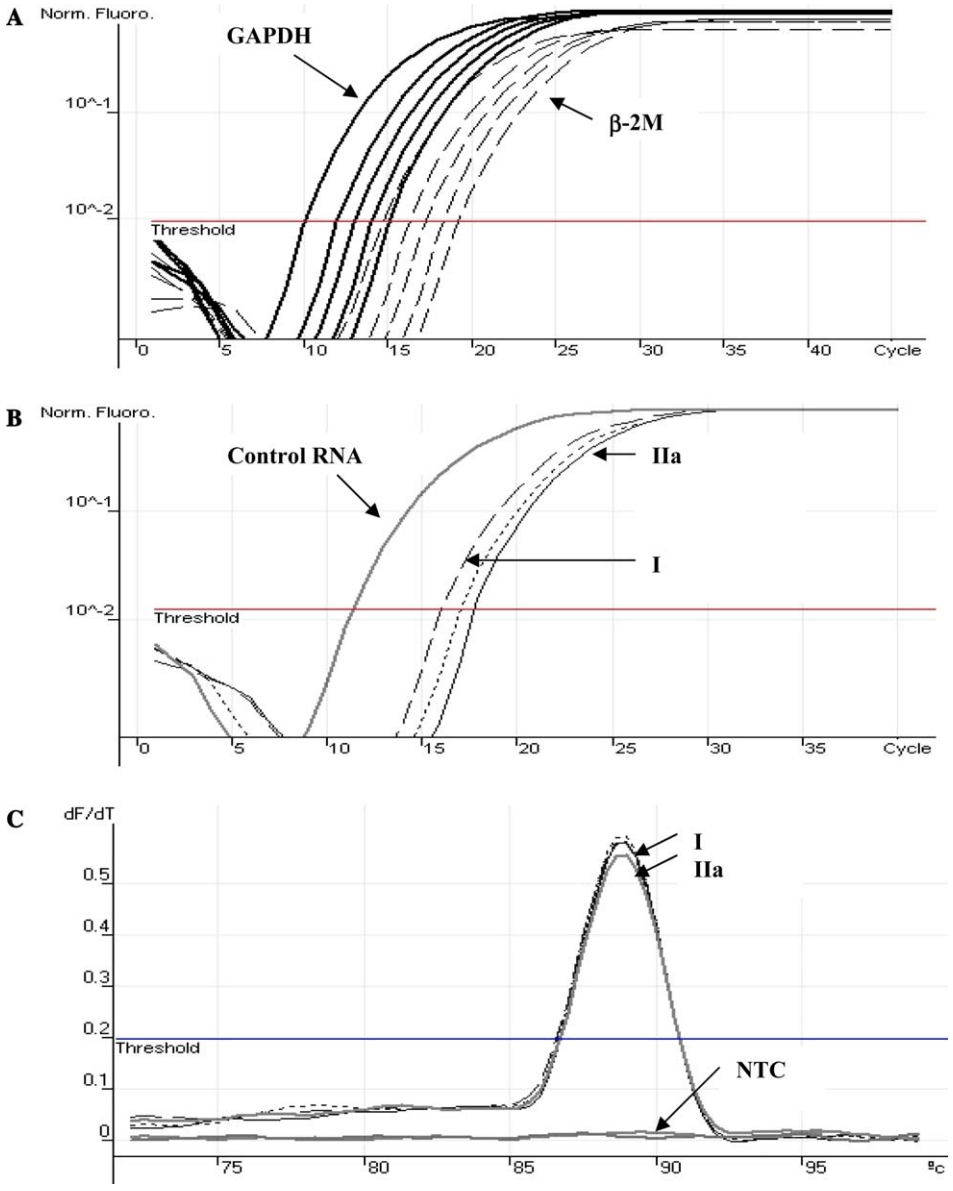

Fig. 2. Amplification time for fluorescence detection and melting curve of PCR product. (A) Curves representing the cycle-dependent fluorescence associated with amplification of GAPDH and β-2M (cDNA) at the concentration of 1.0; 0.5; 0.25; 0.0125; and 0.062 of total RNA are shown. (B) Amplification curves of GAPDH gene from total RNA of MHC I and MHC IIa fibers and control RNA are shown. (C) Typical melting curve (heat dissociation) of PCR product for GAPDH gene in both fiber types. PCR product yields single peak at 88.9 °C.

single-cell mRNA expression. Our results help substantiate these previous findings and extend the use of real-time PCR to single muscle fibers from humans. The muscle biopsy technique has been employed in clinical and sports physiology applications since the early 1960s and is widely used in applied human research. By applying real-time PCR techniques to single muscle fibers, gene expression profiling information can be obtained that is muscle fiber-specific (i.e., slow-twitch vs. fast-twitch). This muscle fiber-specific information may be critical for understanding various molecular alterations at the cell level related to a host of applications such as clinical pathologies, exercise treatments, and nutritional interventions to mention a few. Indeed, both human and animal data have shown that the functional and metabolic profile differs among muscle fiber types [31], highlighting the importance for gene expression information that is muscle fiber-specific.

In summary, data from the current investigation demonstrate that the extraction of high quality RNA from human single muscle fibers along with accurate quantification of selected genes can be reliably performed. These data show that two or more housekeeping genes should be used as internal controls to validate gene expression data from muscle tissue with regard to different muscle fiber types and in response to exercise. This in

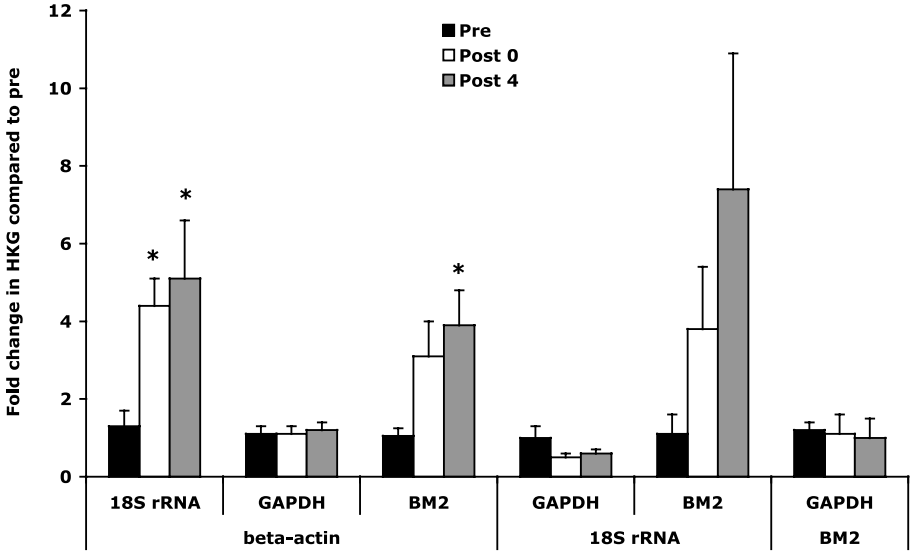

Fig. 3. GAPDH, β-actin, 18S rRNA, and β-2M mRNA content in MHC I single muscle fibers immediately after (Post 0) and 4 h after (Post 4) running exercise. Values are means ± SE. *$p < 0.05$ compared to pre.

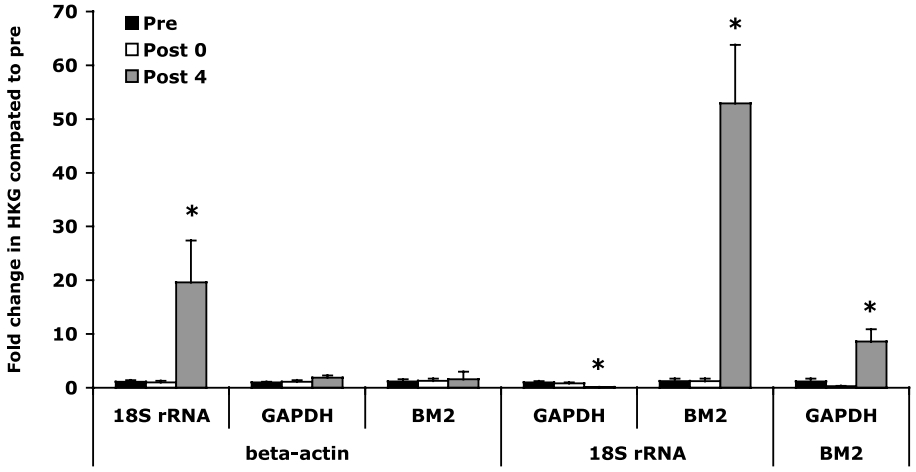

Fig. 4. GAPDH, β-actin, 18S rRNA, and β-2M mRNA content in MHC IIa single muscle fibers immediately after (Post 0) and 4 h after (Post 4) running exercise. Values are means ± SE. *$p < 0.05$ compared to pre.

agreement with other research showing that more than one gene should be used as reference gene to ensure the most reliable transcription analysis [9,28]. When comparing GAPDH, 18S rRNA, β-actin, and β-2M expression in individual muscle fibers, GAPDH can be recommended as the most stable housekeeping gene in response to exercise in slow- and fast-twitch human muscle fibers. The steps performed and presented here provide the basis for future gene expression studies from isolated single muscle fibers obtained from a human muscle biopsy sample.

Acknowledgment

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