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Age-Related Changes in Relative Expression of Real-Time PCR Housekeeping Genes in Human Skeletal Muscle

Chad D. Touchberry,[1] Michael J. Wacker,[1] Scott R. Richmond,[1] Samantha A. Whitman,[1] and Michael P. Godard[2]

[1]Applied Physiology Laboratory, University of Kansas, Department of Health Sport and Exercise Sciences, Lawrence, KS; [2]Department of Kinesiology, Western Illinois University, Macomb, IL

The purpose of this investigation was to examine the expression of three commonly used housekeeping genes—glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β_2-microglobulin (β_2M), and RNA polymerase 2a (polR2a)—in elderly (E) compared to young (Y) subjects. Nine young subjects (22.7 ± 3.4 yrs) and 11 elderly subjects (73.0 ± 9.5 yrs) underwent a percutaneous skeletal muscle biopsy of the *vastus lateralis*. Equal concentrations of isolated mRNA from these samples were used to perform real-time polymerase chain reaction with primer/probe combinations specific to each gene of interest. The expression of GAPDH, β_2M, and polR2a was obtained as the value of cycle threshold (C_T). An independent *t*-test with a level of significance at $p \leq 0.05$ was used to determine differences between groups. There was no difference in average C_T of GAPDH between groups ($p = 0.869$) (Y = 16.92 ± 2.25 vs. E = 17.08 ± 2.09) and polR2a ($p = 0.089$) (Y = 28.00 ± 0.89 vs. E = 26.73 ± 1.91). However, there was a significant difference ($p \leq 0.05$) in the average C_T of β_2M (Y =21.79 ± 0.44 vs. E = 21.05 ± 0.51). The results indicate that special consideration needs to be made when selecting housekeeping genes for comparisons in real-time reverse-transcriptase polymerase chain reaction, depending upon the age of the populations of interest.

Key Words: Housekeeping genes, real-time reverse-transcriptase polymerase chain reaction (RT-PCR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β_2-microglobulin (β_2M), RNA polymerase 2a (polR2a).

Age-related skeletal muscle atrophy (sarcopenia) has severe consequences on the quality of life in elderly populations and also places a significant financial burden on health care systems. It has been estimated that 26 billion dollars of annual health care costs can be attributed to complications directly associated with sarcopenia.[1] Identifying the mechanisms that are directly responsible for these changes in skeletal muscle have been the focus of several laboratories.[2–6] Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) has become a popular means to assess total RNA and mRNA expression, due to its sensitivity, accuracy, and ability to replicate mRNA.[7] Currently, this technique is being used to examine the mRNA expression of specific genes in human skeletal muscle that may be responsible for muscular hypertrophy, as well as muscular atrophy in healthy as well as diseased subjects.[8–15] Not only do the findings of these studies often implicate the genes that are directly involved in regulation of muscle mass, but they also serve to provide new leads for novel therapeutic countermeasures against muscle atrophy.

Housekeeping genes are essential endogenous regulatory genes that are involved in various processes in the cell, such as metabolism, cell structure, gene transcription, and homeostasis, and are therefore constitutively expressed. Glyceraldehyde-3-phosphate dehyrogenase (GAPDH), β_2-microglobulin (β_2M), and RNA polymerase 2a (polR2a) are housekeeping genes that are utilized in studies that implement relative quantitative real-time RT-PCR.[16,17] Recent research has demonstrated that the expression of housekeeping genes may be altered due to differences in tissue types,[18] hypoxia,[19] exercise,[10,13] creatine supplementation,[20] or other experimental treatments.[21,22]

Such data indicate three main issues that need to be addressed when using a housekeeping gene in relative

Address correspondence and reprint requests to: Michael P. Godard, Department of Kinesiology, Western Illinois University, Macomb, IL.

quantitative RT-PCR in human skeletal muscle studies. First, it suggests that housekeeping genes are not completely stable and are open to variation based upon outside influences or disruptions to cells. Second, the stability of the housekeeping gene may be specifically altered, depending upon the independent variables of the investigation. Lastly, these findings clearly indicate that certain housekeeping genes may be less susceptible to alterations from outside influences. These conclusions indicate that some housekeeping genes may better serve as a control when making comparisons to other genes of interest, specifically when there are alterations in the independent variables.

Therefore, it is critical to elucidate the changes, if any, that may exist in these housekeeping genes between younger and older adults. Without this information, age-dependent comparisons are very difficult to make. In using the relative quantitative RT-PCR method, the cycle thresholds of the genes of interest are compared to the housekeeping genes to determine relative changes in expression. Often times, the $\Delta C_{T\ target} - \Delta C_{T\ control}$ ($\Delta\Delta C_T$) is the preferred method of detecting differences in the threshold cycles between the target and control genes. Due to the sensitive nature of the $\Delta\Delta C_T$ method, subtle changes could be interpreted as significant. In fact, the results may be heavily skewed simply due to age-related changes in the specific housekeeping gene being utilized.

In order to address this issue, the housekeeping genes GAPDH (cell metabolism), β_2M (cell structure), and polR2a (gene transcription) were selected for this investigation. These genes are universally expressed and have been used for real-time comparative gene expression studies. In addition, we specifically selected these genes, as they differ in their relative expression level (GAPDH, high expression; β_2M, high to intermediate expression; and polR2a, lower expression), as well as their function within the cell.[17] The purpose of this investigation was to determine any potential differences in the relative expression of these three common housekeeping genes in skeletal muscle between healthy young and healthy elderly populations. The implications of this information can be used to carefully and properly select housekeeping genes that are the most stable when making comparisons to other genes of interest in aging populations.

MATERIALS AND METHODS

Subjects. Twenty healthy subjects—9 young (Y) (22.7 ± 3.4 y; height, 173.97 ± 8.99 cm; weight, 77.52 ± 11.44 kg) and 11 elderly (E) (73.0 ± 9.5 y; height, 167.94 ± 9.37 cm; weight, 74.36 ± 10.34 kg)—were recruited for this investigation. The exclusion criteria for all subjects were obesity (as defined by a Body Mass Index ≥ 30 kg/m^2), any known form of heart disease or hypertension, cigarette smokers, diabetics, or persons taking gluccocorticoids. The research protocol used was in direct accordance with the Declarations of Helsinki and had been approved by the Human Research Committee of the University of Kansas. Prior to giving their consent for participation, all subjects were fully informed of the risks and benefits associated with the muscle biopsies used in this investigation. Written consent was obtained from each subject prior to any testing or data collection.

Muscle biopsy and RNA isolation. Percutaneous muscle biopsies[23] were obtained from the *vastus lateralis* in each subject for analysis. Muscle sections were placed in RNA*later* solution (Ambion, Austin, TX) and then stored at –20°C until analysis. The mRNA was isolated from skeletal muscle biopsies utilizing the μMACS mRNA Isolation Kit (Miltenyi Biotec, Auburn, CA). All mRNA samples were stored at –80°C until analysis.

Real-time RT-PCR. GAPDH, β_2M, and polR2a were analyzed, using standard real-time quantitative RT-PCR. Primers and probes were obtained from ABI (Foster City, California) TaqMan Gene Expression Assay catalog (GAPDH- Hs99999905_m1, ß2M - Hs99999907_m1, polR2A- Hs00172187_m1). These assays come in a 20X reaction mix, span an exon-exon junction, and are optimized to give approximately 100% efficiency. The real-time RT-PCR reactions were performed using a master mix which was created using a SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). All reactions were performed in triplicate using a total of .50 ng of mRNA per reaction. Reactions were set up using an automated liquid handling system (CAS-1200, Corbett Robotics, Sydney, Australia).

Real-time PCR assays were completed using a Rotor-Gene 3000 (Corebett Research, Mortlake, Australia) with a 72-well rotor, following a reverse transcription step for 1 cycle (55°C for 20 min) followed by initial denaturation (95°C for 3 min), then immediately followed by 45 cycles (95°C for 15 sec and 60°C for 45 sec), after which fluorescence was recorded. No-template control tubes (NTC), containing water instead of template mRNA were also run under the same conditions for each gene. Replicate data from all subjects were averaged for each housekeeping gene to conduct comparisons between young and elderly groups. The average C_T value for each housekeeping gene per grouping was then compared to one another (i.e., Young C_T – Elderly $C_T = \Delta C_T$). The formula $2^{\Delta CT}$ was used to calculate the fold change.

Statistical analysis. All statistics were performed using SPSS software program version 12.0 (Chicago, IL). Data

TABLE 1

Differences in Average C_T Value for Each Housekeeping Gene Between Young and Elderly Subjects

Gene	Young (Y)	Elderly (E)	Fold Change
GAPDH	16.92 ± 2.25	17.08 ± 2.09	0.90
β_2M	21.79 ± 0.44[a]	21.05 ± 0.51*	1.67
polR2a	28.00 ± 0.89	26.73 ± 1.91	2.41

Values are means ± SD. [a]Significant difference at $p \leq 0.05$. Fold change indicates difference of elderly gene expression compared to young.

are presented as mean ± standard deviation. Changes in mRNA expression were tested using an independent *t*-test, with a significance level at the $p \leq 0.05$ level.

RESULTS

Housekeeping gene expression in the mRNA extracted from each muscle tissue sample were analyzed via real-time RT-PCR and relative quantification between young and old. A single dilution of mRNA (0.5 ng) was tested with each gene and run in triplicate. Reaction efficiencies of the primer/probe sets were previously determined to be within 5%, and ranged from 97.7 ± 0.9% to 99.4 ± 1.8%; these were previously published.[24] There was no difference in average C_T of GAPDH between groups ($p = 0.869$) (Y = 16.92 ± 2.25 vs. E = 17.08 ± 2.09; 0.90 fold) and polR2a ($p = 0.089$) (Y = 28.00 ± 0.89 vs. E = 26.73 ± 1.91; 2.41 fold). However, in the analysis of β2M, there were significant differences in average C_T ($p = 0.003$) (Y =21.79 ± 0.44 vs. E = 21.05 ± 0.51; 1.67 fold), despite having the least variability from the mean. Table 1 presents the differences in average C_T values, as well as the fold change. Figure 1, panels A, B, and C, present the fluorescence and average cycle number for the three housekeeping genes in each group. In addition, since we were dealing with exponential figures, we performed individual comparisons of the data using $2^{\Delta CT}$, and found the same trend in significance for GAPDH ($p \geq 0.05$), polR2a ($p \geq 0.05$) and β_2M ($p \leq 0.01$).

DISCUSSION

As an increasing volume of data continues to be published exploring mRNA expression in cases of sarcopenia, there has been a greater interest in evaluating the commonly used, widely expressed housekeeping genes for comparisons between young and old. The purpose of this investigation was to examine the validity and reliability of measuring the expression of various housekeeping genes in human skeletal muscle using real-time RT-PCR. To the author's knowledge, this study is the first to report that aging can influence the expression of certain housekeeping genes in human skeletal muscle. Other recent investigations have also seen alterations in housekeeping gene expression in skeletal muscle during various experimental treatments.

Recently, Mahoney et al.[13] investigated the response of housekeeping genes to acute bouts of aerobic (65% of VO_2max) and resistance training exercise (300 eccentric contractions). They noted not only differences in the housekeeping gene expression from exercise at 3 and 48 hours post exercise, but also alterations in the control genes by the type (endurance vs. resistance) of exercise encountered. The authors reported that β_2M and β-actin were the most stable following resistance exercise, while β_2M and GAPDH were most stable following endurance based bouts.

A recent project by Jemiol and Trappe[10] investigated the effect of an acute bout of aerobic exercise (70% of $VO_{2\ max}$) on four endogenous control genes. GAPDH, β-actin, β_2M, and 18s ribosomal RNA were evaluated four hours post exercise from sample biopsies of the gastrocnemius muscle. The findings show that GAPDH remains stable despite an acute bout of endurance exercise. Furthermore, they noted significant changes in β-actin and β_2M following aerobic exercise. The author concluded that exercise does have the potential to disrupt endogenous control genes and that GAPDH appears to be the most stable under cellular disruptions from exercise. While the changes observed in β-actin and β_2M differ from those described by Mahoney et al.,[13] our results suggest that the time course of these changes, as well as variations in exercise intensity, could be of importance.

Interestingly Murphy et al.[20] found that the ingestion of exogenous creatine caused variability in the expression

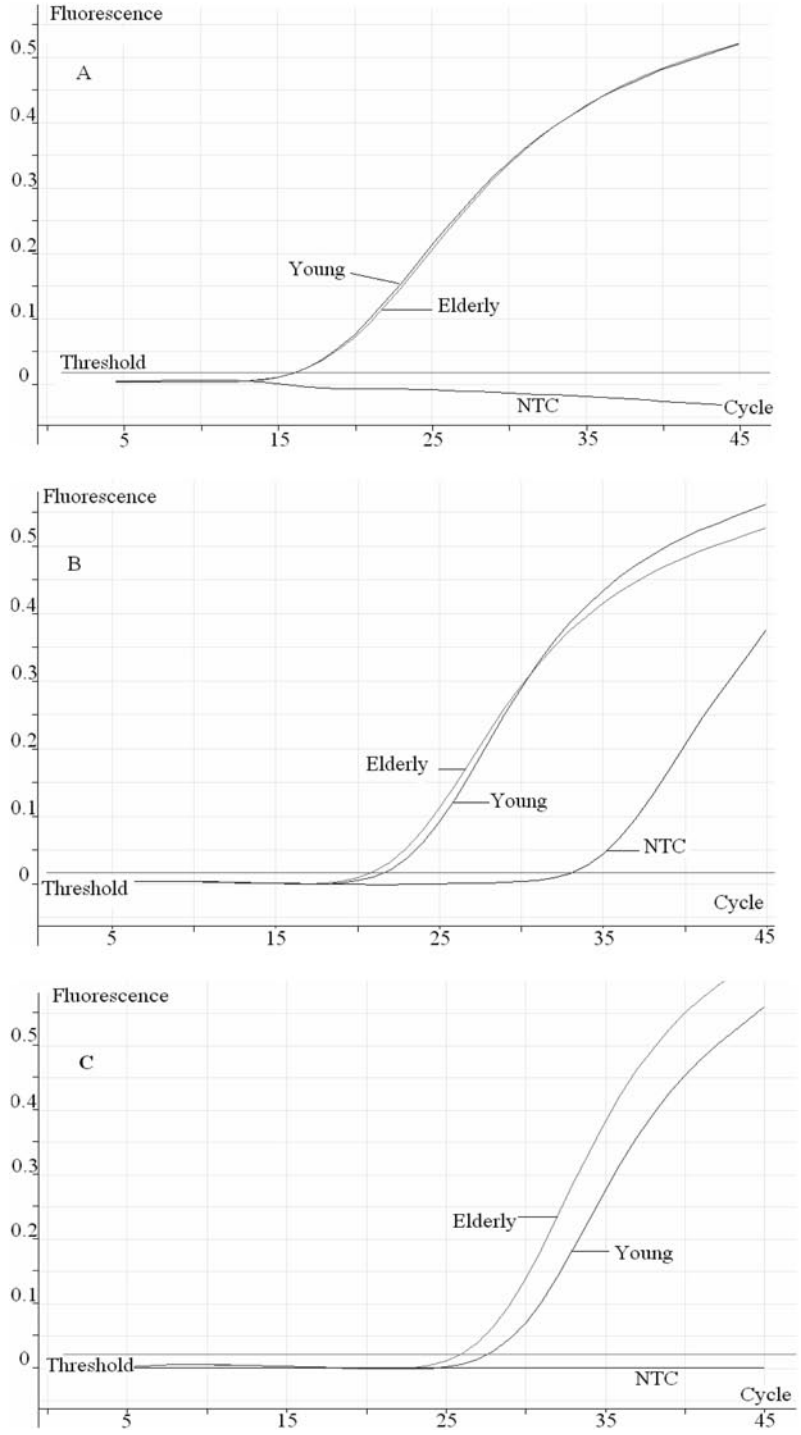

FIGURE 1

Real-time RT-PCR reaction showing the average fluorescence values at each cycle number for young (n = 9) and elderly (n = 11), using GAPDH (**A**), β_2M (**B**) and polR2a (**C**) primers and probes.

of GAPDH and β_2M. These changes were not found to be statistically significant ($p \geq 0.05$), but the genes were described as being less stable than β-actin and recommendations were given against utilizing GAPDH and β_2M for comparisons involving creatine supplementation. However, it is difficult to elucidate whether the changes in expression were a result of the creatine supplementation itself, or the high-intensity, 30-sec cycling bouts that were employed following each muscle biopsy.

Our data are in agreement with the previous findings concerning the stability of these endogenous control genes, in that we also noted alterations in housekeeping genes. Similar to Jemiol and Trappe,[10] we observed that the average expression level of GAPDH had the small-

est difference between old and young groups (Figure 1, panel A). Although our study did not explore the role of exercise, it does add information to a growing body of evidence suggesting that GAPDH is a stable housekeeping gene for between-group comparisons. While any housekeeping gene needs to be tested in all subjects for stability, GAPDH appears to be suitable for skeletal muscle research involving moderate-intensity aerobic exercise as well as aging populations.

Our findings show that when comparing GAPDH, β_2M, and polR2a expression among young and elderly subjects, the suitability of the control gene utilized may depend on what comparisons are being made (i.e., within group vs. between groups). Analyzing GAPDH (Figure 1, panel A), the variation from the mean was larger than the other housekeeping genes ($Y = 16.92 \pm 2.25$ vs. $E = 17.08 \pm 2.09$) between young and old subjects, yet did not approach statistical significance. The variation from the mean of β_2M (Figure 1, panel B) was very small ($Y = 28.00 \pm 0.89$ vs. $E = 26.73 \pm 1.91$) between groups, yet this resulted in a statistically significant difference in expression. polR2a (Figure 1, panel C), on the other hand, had a smaller variation from the mean ($Y = 21.79 \pm 0.44$ vs. $E = 21.05 \pm 0.51$) between groups, and approached statistical significance. In addition, polR2a reached a greater than twofold change in expression between young and elderly groups. It is commonly accepted when quantifying gene expression that a twofold change in expression is significant. It is important that researchers weigh both the statistical differences, as well as the fold change, when making decisions about the stability of housekeeping genes for use within and between groups.

The independent *t*-tests show that GAPDH remains the most stable when comparing across aging populations. GAPDH may not be as suitable a gene for comparisons within groups, due to its large variation from the mean. Furthermore, β_2M and polR2a, due to their small variations within groups, may be suitable for inter-group comparisons when dealing with young and elderly populations, but may not be considered as stable of a housekeeping gene as GAPDH for comparisons between groups.

SUMMARY

This investigation found evidence that there can be variation in the expression of commonly used housekeeping genes with populations of different ages. Given the popularity of real-time RT-PCR and the new influx of data suggesting alterations in mRNA expression of genes that have been labeled as stable for comparison, we feel that there needs to be special consideration given to the selection of housekeeping genes based upon the subject populations used, as well as the use of any therapy. This investigation, as well as the findings of others, has provided critical evidence that housekeeping genes are open to outside influences that may affect their expression.

The findings of our data demonstrate that GAPDH appears to be a more effective gene for group comparisons between young and old populations (Figure 1A), as there was little to no statistically significant variation in the C_T values between groups. However, there did appear to be a trend towards an increased expression of polR2a and β_2M in the elderly. Moreover, researchers may consider using β_2M and polR2a when comparisons within groups are desired, since the variation in their expression level was small within the young and elderly groups. While any housekeeping gene needs to be tested before use in comparative real-time RT-PCR, these results are intended to aid in the proper selection of the housekeeping gene for skeletal muscle and aging studies.

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