Toolbox

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Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise

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Mahoney, Douglas J., Kate Carey, Ming-Hua Fu, Rodney Snow, David Cameron-Smith, Gianni Parise, and Mark A. Tarnopolsky. Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* 18: 226–231, 2004. First published May 25, 2004; 10.1152/physiolgenomics.00067.2004.—Studies examining gene expression with RT-PCR typically normalize their mRNA data to a constitutively expressed housekeeping gene. The validity of a particular housekeeping gene must be determined for each experimental intervention. We examined the expression of various housekeeping genes following an acute bout of endurance (END) or resistance (RES) exercise. Twenty-four healthy subjects performed either a interval-type cycle ergometry workout to exhaustion (~75 min; END) or 300 single-leg eccentric contractions (RES). Muscle biopsies were taken before exercise and 3 h and 48 h following exercise. Real-time RT-PCR was performed on β-actin, cyclophilin (CYC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β2-microglobulin (β2M). In a second study, 10 healthy subjects performed 90 min of cycle ergometry at ~65% of $\dot{V}O_{2\,max}$, and we examined a fifth housekeeping gene, 28S rRNA, and reexamined β2M, from muscle biopsy samples taken immediately postexercise. We showed that CYC increased 48 h following both END and RES exercise (3- and 5-fold, respectively; $P < 0.01$), and 28S rRNA increased immediately following END exercise (2-fold; $P = 0.02$). β-Actin trended toward an increase following END exercise (1.85-fold collapsed across time; $P = 0.13$), and GAPDH trended toward a small yet robust increase at 3 h following RES exercise (1.4-fold; $P = 0.067$). In contrast, β2M was not altered at any time point postexercise. We conclude that β2M and β-actin are the most stably expressed housekeeping genes in skeletal muscle following RES exercise, whereas β2M and GAPDH are the most stably expressed following END exercise.

gene expression; endogenous controls; validation

ANALYSIS of mRNA expression in skeletal muscle has become increasingly prevalent in exercise studies (1, 4, 8, 12, 15, 17, 19, 22). Although numerous techniques are available to estimate mRNA expression, real-time RT-PCR has gained favor among scientists as it is a highly sensitive, accurate, and fast technique that offers high-throughput and the ability to quantify mRNA copy number (10). However, there is substantial technical variability associated with RT-PCR, arising from RNA degradation, differences in the quantity and purity of the input RNA, differences in reverse transcription (RT) and polymerase chain reaction (PCR) efficiency, and pipetting error. PCR exponentially amplifies a small starting quantity of cDNA to a large amount of DNA product; as such, even seemingly insignificant technical variability prior to amplification can lead to considerable error in the end product (7), although this is less of a concern using real-time vs. conventional RT-PCR (10). To overcome this limitation, researchers typically normalize the mRNA expression of their genes of interest to a constitutively expressed internal endogenous control, or housekeeping gene (9). In choosing an appropriate housekeeping gene, several considerations must be taken into account: *1*) the expression of the housekeeping gene must remain constant throughout the given intervention; *2*) the amplification efficiency of the housekeeping gene should be similar to that of the genes of interest; and *3*) the abundance of the housekeeping gene should be similar to that of the genes of interest (6). It is important that a given housekeeping gene be validated for each experimental intervention in which it is used.

There are several common housekeeping genes that have been used to normalize mRNA expression in skeletal muscle following acute exercise, including β-actin (13, 21, 23), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (12, 15, 22), cyclophilin (CYC) (4, 17), and 28S ribosomal RNA (rRNA) (14). Although numerous studies have systematically evaluated the validity of these housekeeping genes following different experimental conditions (11, 18, 26, 27, 29), we are not aware of such a study following acute exercise. Thus the purpose of the present study was to examine whether the genes β-actin, GAPDH, 28S rRNA, CYC, and β2-microglobulin (β2M) are appropriate housekeeping genes following an acute bout of either endurance (END) or resistance (RES) exercise.

MATERIALS AND METHODS

Subjects

The present study used previously collected muscle samples from two larger studies. In *study 1*, 24 healthy males volunteered to participate (age: RES, 23.4 ± 3.7 yr; END, 21.8 ± 2.5 yr). Subjects were assigned to an acute exercise protocol based on their training history, either 300 high-intensity eccentric contractions (RES; $n = 12$) or high-intensity cycle ergometry to exhaustion (~75 min; END; $n = 12$). Subjects in the RES group had not participated in lower body resistance training within 6 mo of the study, and those in the END group had not regularly participated in endurance activities within 6 mo of the study. In *study 2*, 10 healthy, recreationally active men and women (age: 21.9 ± 2.2 yr) were recruited to perform a bout of moderate intensity cycle ergometry (90 min; END; $n = 6$ males, 4 females). Before inclusion into either study, potential subjects were required to complete a health questionnaire to ensure that they were healthy and fit to participate. Each subject was given an information sheet describing all testing procedures, was informed of the purposes and associated risks, and gave written consent prior to participation. The projects were approved by the McMaster University Hamilton

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Health Sciences Human Research Ethics Board and conformed to the Declaration of Helsinki guidelines.

Testing Protocol

Prior to testing, each subject underwent a baseline muscle biopsy collection (see details below). Prior to each tissue collection, subjects were required to abstain from any form of physical exertion (within 72 h), avoid alcohol (within 48 h), eat their habitual diet (within 48 h), and abstain from caffeine (within 12 h). In *study 1*, subjects were given a 355-kcal defined formula diet (Ensure) to be consumed 2 h before each collection, whereas subjects in *study 2* were postabsorptive.

Cycle ergometry protocols (END). Approximately 1 wk following the baseline biopsy, subjects performed a maximal oxygen uptake test ($\dot{V}O_{2\,max}$) using an incremental cycle ergometry test to exhaustion. In *study 1*, no sooner than 72 h following their $\dot{V}O_{2\,max}$ test, subjects performed a high-intensity endurance exercise protocol on the cycle ergometer. Briefly, subjects began pedaling at 60% of their predetermined $\dot{V}O_{2\,max}$ for 30 min at greater than 70 rpm. Following the initial 30 min, the intensity was increased to 65% $\dot{V}O_{2\,max}$ for 5 min, then dropped back to 60% for 5 min, increased to 70% $\dot{V}O_{2\,max}$ for 5 min, dropped to 60% for 5 min, etc., to a maximum of 85% $\dot{V}O_{2\,max}$. If 85% $\dot{V}O_{2\,max}$ was attained, then subjects continued with intervals of 85% $\dot{V}O_{2\,max}$ for 2 min followed by 60% $\dot{V}O_{2\,max}$ for 2 min, etc., until test completion. The test ended when subjects were unable to maintain 70 rpm, which lasted ~75 min for most subjects. In *study 2*, no sooner than 72 h following their $\dot{V}O_{2\,max}$ test, subjects performed 90 min of cycle ergometry at ~65% of $\dot{V}O_{2\,max}$ in the postabsorptive state.

Eccentric contraction protocol (RES). Approximately 1 wk following the baseline biopsy, subjects in the RES group were given a familiarization session with the testing apparatus, a Biodex isokinetic dynamometer (System 3; Biodex Medical Systems, Ronkonkoma, NY). Approximately 1 wk following this session, subjects performed an eccentrically biased isokinetic protocol adapted from one previously described (3). Briefly, following a short warm-up (10 min of light cycle ergometry), subjects were seated in the dynamometer, and their nondominant leg was strapped into a lever arm. The lever arm was programmed to extend the subject's leg to 150° of flexion (where 180° is full extension) at a moderate speed (30°/s), and then flex their leg to 90° of flexion at a fast speed (120°/s). Subjects were not required to contract during the extension phase. During the flexion phase, subjects were instructed to maximally resist the descending lever arm throughout the entire range of motion (i.e., eccentric contraction) and were verbally encouraged to oppose the movement of the lever arm throughout the duration of each contraction. The entire test consisted of 30 sets of 10 repetitions, with a 1-min rest between each set.

Tissue Collection

In *study 1*, muscle biopsies were taken ~1–2 wk before (baseline) and at 3 h and 48 h following the completion of the exercise protocol. In *study 2*, muscle biopsies were taken immediately before and after the exercise protocol. Needle biopsy samples were taken from the vastus lateralis of the nondominant leg ~15 cm proximal to the lateral knee joint of each subject under local anesthesia (1% lidocaine) using manual suction. All biopsies were taken from separate incisions ~6 cm apart, to minimize the potential effect of the biopsy itself on gene expression (20). Approximately 150 mg of muscle was taken from each biopsy, and this was immediately dissected from excess fat and connective tissue. The muscle was quickly sectioned, and ~25 mg was placed in an RNase-free polyethylene tube, flash-frozen in liquid nitrogen, and stored at −86°C until being processed for analysis. The remaining muscle was stored at −86°C for subsequent studies.

Analysis of RNA Expression

RNA extraction. Total RNA was extracted from muscle using a commercially available TRIzol Reagent (Life Technologies, catalog no. 15596) following the manufacturer's instructions. Briefly, ~25 mg of muscle was removed from the freezer and immediately immersed in 1 ml of TRIzol reagent. The muscle was homogenized on ice using a glass homogenizer, and the aqueous and organic phases were separated using 200 μl of chloroform. Total RNA was precipitated using 500 μl of isopropyl alcohol, washed three times with 75% ethanol, redissolved in 15 μl of DEPC-treated H_2O, aliquoted, and stored at −86°C. The concentration and purity of the RNA was determined using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, Ontario) by measuring the absorbance at 260 (OD_{260}) and 280 (OD_{280}) nm. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{280}) of the samples was ~1.4. RNA integrity was assessed in a randomly chosen subset of samples using agarose gel electrophoresis, and the OD ratio of 28S to 18S rRNA was consistently greater than 1 for each sample checked, indicating high-quality RNA.

RT and real-time PCR. For *study 1*, RT was performed on 1 μg of total RNA using a commercially available kit (1st Strand cDNA Synthesis Kit for RT-PCR, Roche, catalog no. 1483188). Prior to RT, RNA samples were treated with DNase I for 30 min at 37°C to remove any contaminating DNA (DNA-free, Ambion, catalog no. 1906) according to the manufacturer's instructions. DNase I was inactivated by a commercially available inactivation solution (DNase inactivation solution, Ambion, catalog no. 1906) according to the manufacturer's instructions. Total RNA was converted to cDNA using AMV reverse transcriptase in a reaction volume of 20 μl containing 1× reaction buffer, 5 mM $MgCl_2$, 1 mM dNTP mixture, 3.2 μg of random primer p(dN)6, 50 U of RNase inhibitor, 0.8 μl of AMV reverse transcriptase, and 1 μg of DNA-free RNA. RT was performed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) with a profile of 25°C for 10 min, 42°C for 60 min, and 99°C for 5 min. All samples were run together, and several negative controls were run simultaneously that did not contain either RNA (no template controls) or the reverse transcriptase enzyme (RT negative), to control for RNA and genomic DNA contamination, respectively. Following RT, samples were individually aliquoted and stored at −80°C until analysis.

For *study 1*, real-time PCR for β-actin, CYC, β2M, and GAPDH was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green 1 chemistry, as previously described (18). This technique has been successfully used to validate housekeeping gene expression levels following serum starvation (25). All samples were run in duplicate. For *study 2*, 28S rRNA and β2M were reverse transcribed and amplified in a single reaction tube and amplification was detected in real time using TaqMan chemistry (TaqMan One-Step RT-PCR Master Mix Reagents, part no. 4309169, Applied Biosystems) according to the manufacturer's instructions. RT (same details as above) and real-time PCR (details in Table 1) were done using an iCycler real-time PCR

Table 1. *PCR details*

Gene	Reaction Volume	Primers	Probes	RNA, ng	PCR Conditions
28S rRNA	50 μl	0.3 μM	0.15 μM	30 ng	1 cycle: 48°C for 30 min, 95°C for 10 min
β2M	50 μl	0.9 μM	0.05 μM	30 ng	30 cycles: 95°C for 15 s, 60°C for 1 min

Real-time PCR was carried out in an iCycler real-time PCR system (Bio-Rad Laboratories). β2M, β2-microglobulin.

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Table 2. *Primer and probe details*

Gene	28S rRNA	β2M
Accession number	M11167	NM004048
Forward primer	cccagtgctctgaatgtcaa	ggctatccagcgtactccaa
Reverse primer	agtgggaatctcgttcatcc	gatgaaacccagacacatagca
Probe	ccttaagagagtcatagttactcccgccgt	acaggaaaactcacgtcatccagcagag
Amplicon length	140 nt	98 nt

Primer sequences were designed using Primer3 (http://www.basic.nwu.edu/biotools/Primer3.html) software on sequences from GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). Primers were checked for mispriming using a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), and free energy of hydrolysis for heterodimers, homodimers, and hairpins was checked using IDT BioTools software (http://biotools.idtdna.com/analyzer/).

system (Bio-Rad Laboratories, Hercules, CA). All samples were run in tetraplicates, fluorescence emission was detected through a Tamra 548 filter, and C_T was automatically calculated. These transcripts were extensively optimized, run simultaneously with RNA- and RT-negative controls, and agarose gel electrophoresis was used to confirm the specificity of the priming.

Primer and probe design. For *study 1*, primers were designed using Primer Express software (Applied Biosystems) as previously described (18). For *study 2*, primers and probes were designed using Primer3 software as described in Table 2.

Statistical Analysis

All statistical analyses were performed on linear data (2^{-CT}) (16). Data from *study 1* were analyzed using a two-way repeated measures ANOVA, with factors of time (PRE, 3 h, 48 h) and group (END, RES). Significant main effects and interactions were further tested using the Tukey honest significant difference (HSD) post-hoc test. Data from *study 2* were analyzed using a dependent *t*-test. All data are expressed as mean fold change from PRE (±SE), using $2^{-\Delta CT}$. All analyses were done using computerized statistics software (Statistica; Statsoft, Tulsa, OK).

RESULTS

Real-Time RT-PCR Quality Control and Performance Characteristics

After optimization, the amplification for all genes was shown to be specific and highly efficient. Quality control data and performance characteristics for β-actin, CYC, β2M, and GAPDH have been presented elsewhere (18). For 28S and β2M analysis using TaqMan chemistry and a one-step protocol, real-time RT-PCR was optimized and monitored to have combined RT and PCR efficiencies of 110% (r^2 = 0.98) and 113% (r^2 = 0.97), respectively, and intra-assay CV values of 16% and 17%, respectively (using linear 2^{-CT} values). Agarose gel electrophoresis confirmed that a single, specific product of appropriate size was being amplified. After 40 cycles of PCR, there was no observable amplification in the RNA or RT-negative control. For quality control, we compared the data generated using real-time RT-PCR with conventional agarose gel detection RT-PCR (using β-actin) and found a correlation coefficient of 0.99 ($P < 0.01$; not shown).

Analysis of Housekeeping Genes

From *study 1*, we analyzed the housekeeping gene data using both nonnormalized C_T values ($2^{-\Delta CT}$) and C_T values of each individual housekeeping gene normalized to each other ($2^{-\Delta\Delta CT}$). The latter approach was taken to observe the relationship between the various housekeeping genes. Using nonnormalized $2^{-\Delta CT}$ values, we showed that CYC increased at 48 h following both END and RES exercise (3- and 5-fold, respectively; $P < 0.01$; Figs. 1 and 2) and was variable in the RES group at 3 h. β-Actin trended toward an increase following END exercise (1.85-fold collapsed across time; $P = 0.13$;

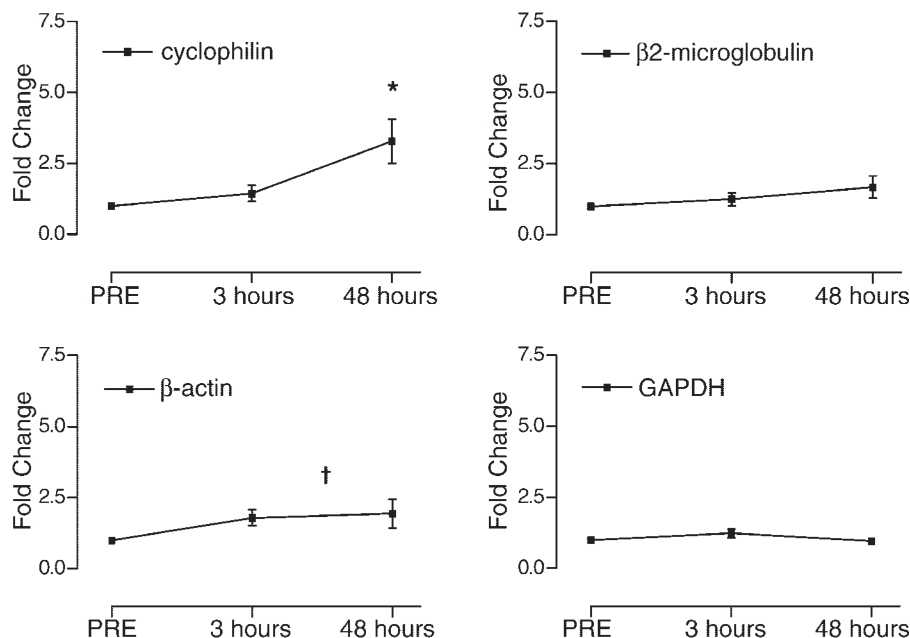

Fig. 1. mRNA expression of β-actin, cyclophilin (CYC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β2-microglobulin (β2M) following endurance (END) exercise. CYC increased 48 h following END exercise (3-fold; *$P < 0.01$), whereas β-actin trended toward an increase at 3 h and 48 h (1.85-fold collapsed across time; †$P = 0.13$). GAPDH and β2M were not altered at either time point. Values are means ± SE.

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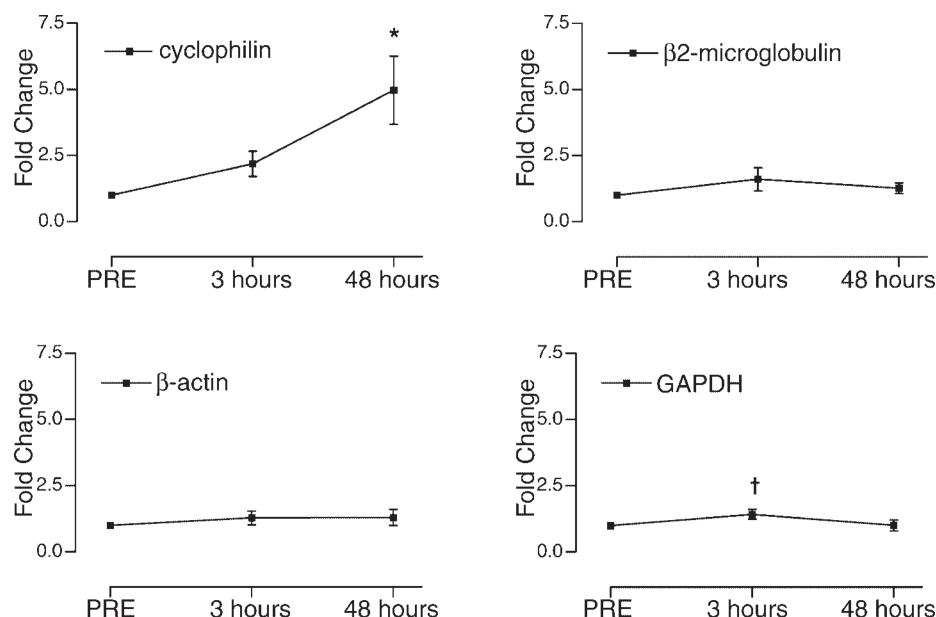

Fig. 2. mRNA expression of β-actin, CYC, GAPDH, and β2M following resistance (RES) exercise. CYC increased 48 h following RES exercise (5-fold; *P < 0.01), whereas GAPDH trended toward a small yet robust increase at 3 h (1.4-fold; †P = 0.067). β2M and β-actin were not altered at either time point. Values are means ± SE.

Fig. 1), whereas GAPDH trended toward a small but constitutive increase at 3 h following RES exercise (1.4-fold; P = 0.067; Fig. 2). In contrast, β2M was not significantly altered post-END or post-RES exercise, and β-actin and GAPDH were very stable in the RES and END groups, respectively (Fig. 1 and 2). When we examined each housekeeping gene normalized to one another ($2^{-\Delta\Delta CT}$), we found similar results (data not shown). Finally, from *study 2* we found that 28S rRNA significantly increased immediately following END exercise (2-fold; P = 0.02; Fig. 3), whereas β2M was stable (P > 0.05; Fig. 3).

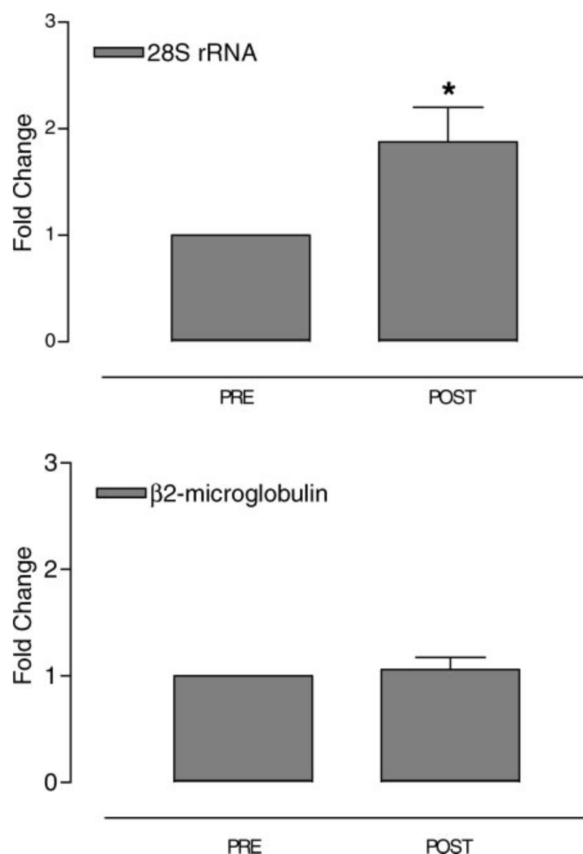

Fig. 3. Expression of 28S ribosomal RNA (rRNA) and β2M mRNA immediately following a 90 min bout of moderate intensity (~65% $\dot{V}O_{2max}$) exercise. 28S rRNA increased ~2-fold immediately following END exercise (*P = 0.02; n = 10), whereas β2M expression remained stable immediately post-END exercise. Values are means ± SE.

DISCUSSION

As there are an increasing number of studies exploring mRNA expression in skeletal muscle following acute END and RES exercise (4, 12–15, 17, 21–23), we were interested in systematically evaluating several commonly used housekeeping genes following an acute bout of END and RES exercise. Following our exercise bouts, β2M and GAPDH were stably expressed following END exercise, whereas β2M and β-actin were stably expressed following RES exercise. In contrast, CYC was significantly altered following both END and RES exercise, 28S rRNA was significantly altered immediately following END exercise, and GAPDH and β-actin were very

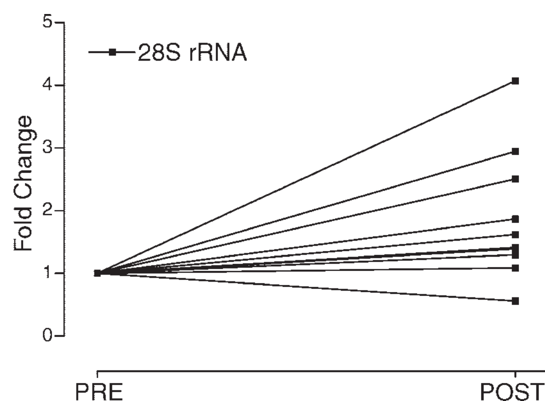

Fig. 4. 28S rRNA response variability following END exercise. Although the direction of the response was similar for 9 of 10 subjects, there was substantial variance in the degree to which each subject responded.

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unstable following RES and END exercise, respectively. Together, we feel that β2M and GAPDH are appropriate housekeeping genes following END exercise, whereas β2M and β-actin are appropriate following RES exercise.

Several investigators have used CYC as a housekeeping gene following acute exercise (4, 17), as well as β-actin following acute END exercise (13, 21, 23) and GAPDH following acute RES exercise (15, 22). Our data suggest that these may not have been appropriate housekeeping genes to use following such interventions. Given that we observed increases in each of these "housekeeping" genes post-END and/or post-RES exercise, it is possible that small increases in mRNA expression of the genes of interest may have been "masked" in these studies, and observable changes may have, in reality, been larger than reported. However, it should be noted that several issues ought to be considered before making broad extrapolations of our data to the literature. First, the intensity of our exercise protocols were very high, and intensity is likely a determinant of gene expression in skeletal muscle post-acute exercise, as has been shown postexercise training (24). Second, our RES protocol consisted solely of eccentric contractions, and it is possible that CYC and GAPDH may have been more stably expressed following dynamic resistance exercise with a concentric component, as eccentric and concentric contractions are known to induce different molecular responses (5). Finally, our study is limited in the number of time points examined postexercise. As gene expression can be rapidly altered following exercise, our results at 3 h and 48 h may not represent all 48 h following exercise.

Intra- and Interindividual Variability

Recently, two studies have reported large intra- and interindividual variability in skeletal muscle gene expression using DNA microarrays (2, 28). We have not previously observed much intra-individual variability using many of the same genes examined in the present study (18). Furthermore, we used a repeated-measures design, which compared individuals to themselves but not to one another, thereby eliminating the interindividual variability in the resting state. However, it is possible that the genetic or epigenetic differences among our subjects may have affected their response to the exercise bout. To manage these differences, we recruited a relatively homogenous group of subjects and controlled for potentially confounding variables such as training status, medical history, pre-exercise diet, and alcohol consumption. Despite this, we observed a variable response to both protocols between individuals for all genes tested. For example, with 28S rRNA, although 9 of 10 subjects responded in the same direction following END exercise, each individual subject responded to a highly variable degree (CV of the response = 55%; Fig. 4). Such variability strengthens any statistically significant changes that we observed (e.g., CYC), but in general would have made it more difficult to observe a statistically significant difference in gene expression. Interestingly, this variance appeared to be somewhat gene specific, as we were nearly able to significantly detect a 1.4-fold increase in GAPDH ($P = 0.067$), but were less able to significantly detect a larger 1.85-fold increase in β-actin ($P = 0.13$). Large "response variability" in gene expression following exercise has been previously documented (22), and the genetic or epigenetic mechanism behind this phenomenon is beyond the scope of this paper.

Conclusion

Given the constitutive expression, moderate abundance, and high RT-PCR efficiency, we conclude that β2M and GAPDH are appropriate housekeeping genes for studies of mRNA expression in skeletal muscle following acute END exercise, whereas β2M and β-actin are appropriate housekeeping genes for studies of mRNA expression in skeletal muscle following acute RES exercise. Furthermore, given the unstable expression postintervention, we feel that CYC is not a valid housekeeping gene in skeletal muscle following both END and RES exercise, 28S rRNA is not appropriate following END exercise, and GAPDH and β-actin are unsuitable following RES and END exercise, respectively. However, this study utilized very intense, specific exercise protocols and examined limited time points postexercise; as such, we caution investigators against simply extrapolating these results across all exercise protocols. Although we feel that our results should serve as a "guide" for choosing a housekeeping gene in future studies examining gene expression in skeletal muscle postexercise, it is imperative that the appropriateness of a given housekeeping gene be examined for each exercise protocol.

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GRANTS

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