Elevation in heat shock protein 72 mRNA following contractions in isolated single skeletal muscle fibers

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Stary CM, Walsh BJ, Knapp AE, Brafman D, Hogan MC. Elevation in heat shock protein 72 mRNA following contractions in isolated single skeletal muscle fibers. Am J Physiol Regul Integr Comp Physiol 295: R642–R648, 2008. First published June 4, 2008; doi:10.1152/ajpregu.00852.2007.—The purpose of the present study was 1) to develop a stable model for measuring contraction-induced elevations in mRNA in single skeletal muscle fibers and 2) to utilize this model to investigate the response of heat shock protein 72 (HSP72) mRNA following an acute bout of fatiguing contractions. Living, intact skeletal muscle fibers were microdissected from lumbrical muscle of *Xenopus laevis* and either electrically stimulated for 15 min of tetanic contractions (EX; n = 26) or not stimulated to contract (REST; n = 14). The relative mean developed tension of EX fibers decreased to 29 ± 7% of initial peak tension at the stimulation end point. Following treatment, individual fibers were allowed to recover for 1 (n = 9), 2 (n = 8), or 4 h (n = 9) prior to isolation of total cellular mRNA. HSP72, HSP60, and cardiac α-actin mRNA content were then assessed in individual fibers using quantitative PCR detection. Relative HSP72 mRNA content was significantly (P < 0.05) elevated at the 2-h postcontraction time point relative to REST fibers when normalized to either HSP60 (18.5 ± 7.5-fold) or cardiac α-actin (14.7 ± 4.3-fold), although not at the 1- or 4-h time points. These data indicate that 1) extraction of RNA followed by relative quantification of mRNA of select genes in isolated single skeletal muscle fibers can be reliably performed, 2) HSP60 and cardiac α-actin are suitable endogenous normalizing genes in skeletal muscle following contractions, and 3) a significantly elevated content of HSP72 mRNA is detectable in skeletal muscle 2 h after a single bout of fatiguing contractions, despite minimal temperature changes and without influence from extracellular sources.

Exercise; heat shock; stress

IT HAS BEEN WELL DOCUMENTED that stimuli that disrupt intracellular homeostasis, such as thermal elevation, ischemia-reperfusion, alterations in pH, and application of free radicals, can induce the transcription of a highly conserved group of cytoprotective elements termed heat shock proteins (HSPs) (28, 34, 46). A prominent and well-studied HSP is the 72-kDa protein HSP72 (8, 11, 17), which has been shown to prevent protein aggregation and degradation during conditions of cell stress, thereby promoting subsequent recovery by protection and restoration of dysfunctional enzymes in a variety of tissues including cardiac (33, 34, 54, 61) and skeletal muscle (34, 36).

Exercise is a nonspecific stress that particularly affects skeletal muscle, and it has been established in whole animal (57, 60) and human models (13, 47, 53, 69, 71) that a single bout of high-intensity exercise can induce an acute elevation in skeletal muscle HSP72. However, due to the multiplicity of potential signaling mechanisms that occur simultaneously within intensely contracting skeletal muscle, including alterations of the phosphorylation potential (12, 23, 50), impaired cytosolic Ca^{2+} cycling (76, 78), decreases in intracellular PO_{2} (21, 56), accumulation of metabolic byproducts in the extracellular medium, and the generation of heat, which may reach 45°C in contracting whole muscle (57), it remains uncertain which, if any, intracellular disruptions may be responsible for the observed increased production of HSP72. Furthermore, the utilization of whole animal and isolated muscle preparations make it difficult to isolate and determine the precise signaling mechanisms for induction of HSP72 at the cellular level.

Unlike whole animal and whole muscle models, in the isolated intact single skeletal muscle fiber preparation the extracellular environment is homogeneous and can be easily adjusted and determined, therefore eliminating complications associated with extracellular stimuli, intracellular substrate availability, extracellular pH, and/or metabolic waste product removal. In addition, through conduction and convection in a well-stirred medium, the minimal amount of internal labile heat produced from contractions (31) is rapidly dissipated, and temperature is therefore predominantly determined by the extracellular environment, which can be easily set and maintained. Therefore, to determine whether an exercise-induced intracellular signal other than heat production can stimulate an immediate elevation of HSP72 mRNA in skeletal muscle, we developed a stable model for measuring contraction-induced elevations in mRNA in single skeletal muscle fibers via quantitative PCR (qPCR) utilizing TaqMan minor-groove binder (MGB) probes. We then employed this model to test the hypothesis that a single bout of fatiguing, tetanic contractions would induce an acute elevation of HSP72 mRNA in isolated single skeletal muscle fibers at 1, 2, and 4 h.

Methods

Female adult *Xenopus laevis* were used in this investigation. All procedures were approved by the University of California San Diego Animal Use and Care Committee and conform to National Institutes of Health standards.

Experimental protocol. Single muscle fibers were isolated and prepared as described previously (22). Briefly, frogs were doubly pithed, and the lumbrical muscles (II-IV) were microdissected with tendons intact in a chamber of physiological Ringers solution at a pH = 7.0. Platinum clips

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were attached to the tendons of each muscle fiber to facilitate positioning within the Ringers solution-filled chamber. One tendon was fixed, whereas the other tendon was attached to an adjustable force transducer (model 400A; Aurora Scientific, Aurora, Ontario, Canada), allowing the muscle to be set at optimum muscle length. The analog signal from the force transducer was recorded via a data acquisition system (AqcKnowledge; Biopac Systems, Santa Barbara, CA) for subsequent analysis. Individual fibers were perfused throughout the experiment with air-equilibrated Ringers solution to maintain a stable temperature (22°C) and to reduce the occurrence of an appreciable unstirred layer surrounding the cell.

Immediately following microdissection, individual fibers were randomly assigned to either the contraction treatment group (EX; n = 26), consisting of 15 min of tetanic contractions at a 0.33-Hz stimulation frequency, or to the no-contraction treatment group (REST; n = 14). REST fibers were kept in solution following microdissection for either 0 (n = 4), 1 (n = 3), 2 (n = 4), or 4 h (n = 3). Tetanic contractions were elicited using direct (8–10 V) stimulation of the muscle (model S48; Grass Instruments, Warwick, RI). The stimulation protocol consisted of ~250-ms trains of 70-Hz impulses of 1 ms duration. Following contractions, fibers were processed for isolation of total cellular RNA at either 1 h (n = 9), 2 h (n = 8), or 4 h (n = 9) posttreatment. Prior to processing for RNA isolation, individual fibers were electrically stimulated to test for fiber viability.

**RNA extraction and cDNA synthesis.** Isolation of total cellular RNA from individual fibers was performed using a protocol incorporating Trizol reagent (Invitrogen, Carlsbad, CA) and the Micro RNeasy Total RNA isolation kit (Qiagen, Valencia, CA). Individual fibers were manually cleaned of residual cellular debris and removed from the tendon. Fibers were then introduced into 0.5 ml of Trizol reagent, and RNA was isolated according to the manufacturer’s protocol. RNA was then eluted using the Micro RNeasy Total RNA isolation kit (Qiagen). Quantification of RNA content of 14 separate fibers was performed in triplicate using a NanoDrop fluorospectrometer (model ND-3300) with Ribogreen RNA fluorescent indicator dye (NanoDrop, Wilmington, DE). The amount of total mRNA recovered from these single fibers was 14 ± 2 pg·μl⁻¹·fiber⁻¹. First-strand cDNA synthesis was performed on the DNase-treated total RNA using a ThermoScript Taq-free kit (Invitrogen) per manufacturer’s protocol. All resulting cDNA was subjected to qPCR amplification using TaqMan MGB probes with 6-FAM fluorescence detection.

**TaqMan-MGB primers and fluorogenic probe design.** Taq-Man-MGB probes (Applied Biosystems, Foster City, CA) are a new class of reporter probes that incorporate a 5′ reporter dye (6-FAM) and a 3′ nonfluorescent quencher (30), offering the advantage of lower background signal and increased melting temperature, thereby increasing sequence specificity. Sequence-specific TaqMan primers and probes were designed to definitive *Xenopus laevis* gene sequences recorded in GenBank for HSP72 (GenBank accession no. BC078115), HSP60 (GenBank accession no. BC041192), and cardiac α-actin (GenBank accession no. X03469) with Primer Express version 2.0 (Applied Biosystems). Optimal primers and probes were 20–80% GC rich, between 9–40 bases in length, had primer melting temperature values of 58–60°C, with probe melting temperatures 10°C higher than the primer melting temperatures. The probe selected was close to the 3′ end of the forward primer, had more bases of Cs than Gs, and < 4 contiguous Gs in the strand, as recommended by the manufacturer and described by Livak et al. (37). Amplicons were then homology-searched to ensure that they were specific for the target mRNA transcript using an NCBI BLAST search. Primers and probe sequences are presented in Table 1.

**Real-time qPCR with TaqMan-MGB probes.** qPCR was performed using an MX3000P real-time PCR system (Stratagene, La Jolla, CA) incorporating TaqMan 6-FAM detection assays. Each reaction contained 15 μl of 2× TaqMan Master Mix (Applied Biosystems), 0.27 μl ROX reference dye (Stratagene) diluted 1:200 with RNase-free H₂O, 0.9 μl of 10 μM forward and reverse sequence detection primers (Applied Biosystems), and 2.0 μl cDNA brought to a final volume of 30 μl with ultrapurified H₂O. In addition, no-template control and no-reverse transcription control (DNase-treated total RNA not subjected to cDNA synthesis) reactions were included to test for any degree of contaminating genomic DNA in the RNA or reaction samples and/or primer-dimer amplification.

Amplification was performed in 0.6 ml 96-well polypropylene real-time PCR plates (Stratagene). qPCR parameters were as follows: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. As the comparative quantification calculation is based on the assumption that amplification of individual genes occurs with similar reaction efficiencies, qPCR assays of three-step 10× serial dilutions of RT product from heat-shocked (37°C for 1 h; n = 6) *Xenopus* muscle bundles were performed with each gene primer set used, and the reaction efficiencies calculated and compared. Heat shock was sufficient to generate sufficient HSP72 mRNA content for an accurate serial dilution. Following amplification, select amplicons were electrophoresed on a 1.5% agarose gel adjacent to a 100-bp ladder, and visualized with ethidium-bromide for fragment size. Band location of amplicon for HSP72, HSP60, and cardiac α-actin corresponded to sequence size (~60 bp), indicating specific amplification of the desired sequence. No bands were detected in the negative control no-template control or no-reverse transcription control lanes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>TaqMan-MGB Probe Sequence</th>
<th>TaqMan-MGB Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP72</td>
<td>+ Sense</td>
<td>TCCAAGTGTGTAGCCGAT</td>
<td>TGATGTTGCAATGTGACTTGGAAAGCA</td>
</tr>
<tr>
<td>HSP72</td>
<td>- Sense</td>
<td>TCCACTGTTTATCCAGCTTCAGCTT</td>
<td>TTTGTCTTATCTACCTTGGACCTTT</td>
</tr>
<tr>
<td>HSP60</td>
<td>+ Sense</td>
<td>CCCAAGGGGAAAGAC</td>
<td>GATCTGCTGGTGCTGAAATG</td>
</tr>
<tr>
<td>HSP60</td>
<td>- Sense</td>
<td>ATCTGCGGGGCTGCGGGGAGA</td>
<td>AATCGCTCGGCTGCGGGGAGA</td>
</tr>
<tr>
<td>Cardiac α-actin</td>
<td>+ Sense</td>
<td>CATGGATTCGTCTCCGGTAC</td>
<td>GGCTGACGCCAGCCTTGCTG</td>
</tr>
<tr>
<td>Cardiac α-actin</td>
<td>- Sense</td>
<td>CAGCTGATTCGTCTCCGGTAC</td>
<td>CAGCTGATTCGTCTCCGGTAC</td>
</tr>
</tbody>
</table>

MGB, minor-groove binder; HSP, heat shock protein.
indicating that these samples were free from both genomic DNA contamination and amplified primer-dimer.

Calculations. The suitability of cardiac α-actin and HSP60 as internal, endogenous normalizers, i.e., housekeeping genes (HKGs), and the fold change of HSP72 mRNA between treatment groups were determined by the relative quantification method (ΔΔCt), as originally reported by Livak and Schmittgen (38), according to the equation, where ΔΔCt = (Ct target − Ct reference) treatment 1 − (Ct target − Ct reference) treatment 2, where Ct is the threshold cycle, and the target gene in each treatment group is normalized to the reference gene. To compare the suitability of HSP60 and cardiac α-actin as HKGs, the fold change in single fibers from the contraction treatment group (treatment 2) of HSP60 mRNA (target) was calculated with cardiac α-actin as the reference (HKG) gene and relative to the expression of HSP60 in single fibers from the rest treatment group (treatment 1). Using this analysis, if the level of the two 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 20 = 1) (38). In a similar manner, the fold change of HSP72 of single fibers in the contraction treatment group was analyzed with either HSP60 or cardiac α-actin as the reference gene and relative to the expression of HSP72 in single fibers from the rest treatment group. An increase in HSP72 mRNA resulting from the treatment effect will be reflected by values of fold change > 1.

Data and statistical analysis. Changes in force over time were tested via a repeated-measures two-way ANOVA. Relative fold change in qPCR amplicon content between treatment groups was tested via a two-way ANOVA. When significant F values were present, the Bonferroni post hoc test was employed for determination of between-group differences. Relative fold changes from mean rest values in qPCR amplicon content were tested via a one-sample t-test, with 1 as the test variable, since 20 = 1. Statistical significance was accepted at P < 0.05.

RESULTS

Examples of fluorescence emission during PCR amplification for 10× serial dilutions of RT product from a heat-shocked Xenopus skeletal muscle bundle are illustrated in Fig. 1 for HSP60 (Fig. 1A), cardiac α-actin (Fig. 1B), and HSP72 (Fig. 1C). When the Ct of individual TaqMan primer/probe sets are plotted against the base 10 log of the dilution, (Fig. 1D), individual amplification efficiencies of RT product from this bundle for each primer/probe set can be calculated. The mean amplification efficiencies for all serial dilutions (n = 3) of individual TaqMan primer/probe sets yielded a mean efficiency of 101.7 ± 2.3% (HSP60 = 99%; cardiac α-actin = 101%; HSP72 = 106%), indicating that these primer/probe sets are suitable for comparative Ct analysis of their respective genes.

In fibers subjected to 15 min of 0.33 Hz tetanic contractions (EX fibers), force fell significantly (P < 0.05, Fig. 2) in all fibers to 29.3 ± 6.7% of initial developed tension, with no difference between 1-, 2-, or 4-h groups at the fatigue end point, demonstrating development of fatigue. Mean cross-sectional area of all fibers was 285 ± 13 μm². The development of fatigue and cross-section measurements suggests that the fibers used in the present study were primarily fast, glycolytic fibers (64).

The mean fold change of HSP60 mRNA normalized to cardiac α-actin for all single fibers from the EX groups compared with fibers from the REST treatment group is shown for each time point in Fig. 3A. No significant difference (P < 0.05)
section did not significantly induce HSP72 transcription in these single skeletal muscle fibers (data not shown).

**Discussion**

The results of the present study, in which isolated single skeletal muscle fibers were subjected to an acute bout of fatiguing contractions followed by comparative analysis of HSP72, HSP60, and cardiac α-actin mRNA demonstrate that 1) isolation of mRNA followed by qPCR can be reliably performed in isolated single skeletal muscle fibers, 2) HSP60 and cardiac α-actin are suitable HKGs for comparative analysis of transcription in skeletal muscle in response to contractions, 3) HSP72 mRNA content significantly increases in these single skeletal muscle fibers 2 h following a single bout of fatiguing contractions, and 4) the contraction-related stimulus for increased HSP72 mRNA in these skeletal muscle fibers is intracellular in nature, and occurs independently of any significant alteration in temperature.

**Single skeletal muscle fiber model of transcriptional activation following contractions.** The upregulation of HSP72 occurs following activation and trimerization of heat-shock transcription factor, permitting binding to the heat-shock response promoter element with subsequent initiation of transcription (2). Although HSP72 mRNA expression is regulated following activation of heat-shock transcription factor (7), protein expression appears to be controlled at the level of transcription (51, 58, 73). Increased transcription of HSP72 mRNA in skeletal muscle following a single, acute bout of exercise has been previously described in both exercising animals (20, 39, 43, 44, 57, 60) and humans (15, 26, 27, 47, 52, 55, 56–59, 67–69, 72). However, an accurate assessment of the cellular transcriptional response of skeletal muscle to contractions in these models is precluded by heterogeneity of tissue, motor unit recruitment, and muscle fiber type. Furthermore, due to the heterogeneous mix of tissue contained in whole muscle or biopsy homogenates which have been shown to produce HSP72 in response to stress, including nervous tissue (59), endothelium (25), and phagocytic cells (27), the increase in HSP72 mRNA arising from the skeletal muscle myocyte alone cannot be established from these previous studies.

The isolated single skeletal muscle fiber preparation used in the present study is free from these above-mentioned constraints and was therefore utilized to establish a model of gene analysis following contractions in skeletal muscle. Isolated single skeletal muscle fibers from *Xenopus laevis* have been

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**Figure 2.** Representative stress (developed tension/cross-sectional area) recording of an individual fiber (A) and relative mean peak developed tension of all fibers subjected to 15 min of 0.33-Hz tetanic contractions (B; means ± SE; n = 26). Significant development of fatigue was demonstrated at the stimulation end point in all fibers; however, no difference in relative developed tension was observed at the stimulation end point between fibers allowed to recover 1, 2, or 4 h.

**Figure 3.** Relative mean fold change from rest of HSP60 mRNA normalized to cardiac α-actin (A) and relative mean fold change from rest of HSP72 mRNA normalized to HSP60 and to cardiac α-actin (B). HSP72 mRNA was significantly (P < 0.05) elevated at the 2-h time point, relative to rest fibers (B). *Significant increase above rest (>1-fold, since 2^0 = 1).
commonly employed in delineating mechanisms of fatigue (74, 75, 77) and muscle bioenergetics during contractions (21, 50, 62, 63). Furthermore, the Xenopus HSP72 gene has been cloned (5, 29), and it has been demonstrated that Xenopus skeletal muscle responds to heat stress with increased transcription of HSP72 (1). However, prior to the present study, it was unknown whether Xenopus skeletal muscle increases transcription of HSP72 mRNA in response to contractions, as has been documented in other species.

In the present study, the limited amount of total mRNA recovered from these single fibers prevents utilization of the standard dilution technique of qPCR analyses of mRNA content. For example, while the standard dilution technique, in which the absolute quantification of starting material is determined through calculations of reaction efficiency (determined from serial dilutions of known concentrations of genetic material), provides an estimate of absolute copy count of starting genetic material, comparisons between samples is improved with a standardized amount of genetic material (9). However, relative increases in transcription of select genes from human skeletal muscle fiber segments has recently been measured using relative quantification analyses of real-time PCR amplicons (24, 38). This technique utilizes an endogenous normalizing gene to standardize the number of duplicative cycles necessary to achieve a given level of fluorescence (Ct), and thereby the relative quantity of starting material, between samples.

**Endogenous normalizers (HKGs).** A commonly used endogenous control gene in skeletal muscle is the glycolytic enzyme GAPDH. However, it has been demonstrated that the expression of GAPDH mRNA may become unstable following exercise paradigms (41, 49), suggesting that its use as a HKG may be inappropriate in studies investigating the transcriptional response to exercise. A similar debate exists over the common use of the skeletal muscle structural protein β-actin as a suitable HKG in skeletal muscle following exercise (24, 41), suggesting that the frequent use of these genes as endogenous internal controls may be inappropriate in studies incorporating skeletal muscle contraction. In the present study, cardiac α-actin and HSP60 were employed as novel internal HKGs in skeletal muscle following contractions.

Cardiac α-actin and skeletal muscle α-actin are thin filament variants of the contractile apparatus that are coexpressed in both heart and skeletal muscle (18, 70), yet are likely differentially regulated (18, 19). Although stable expression of cardiac α-actin following exercise has been demonstrated in cardiac muscle (66), the results of the present study are the first to employ cardiac α-actin as an endogenous control in single skeletal myocyte gene expression assays. HSP60 is a component of mitochondrial chaperonin, the major site of mitochondrial protein folding for import and increased expression has been demonstrated to preserve mitochondrial function and to diminish apoptosis in cardiac myocytes following ischemia-reperfusion (35). While HSP60 protein has been shown to increase in muscle many hours to days following exhaustive exercise (26, 47), skeletal muscle HSP60 mRNA has been shown to be stable 2 h postexercise (14). In the present study, the relative ratio of HSP60 mRNA to cardiac α-actin mRNA in isolated single skeletal muscle fibers remained unchanged at all time points following 15-min tetanic fatiguing contractions (relative to REST fibers, Fig. 3A), demonstrating the potential suitability of either gene as a general internal control for studies investigating the acute transcriptional response of skeletal muscle to contractions.

**HSP72 mRNA in response to contractions.** Similar to the stable expression of HSP60, the expression of HSP72 mRNA remained stable in all REST fibers, independent of postdissection recovery time, indicating that any possible transcriptional activation induced by stretch (6, 79) or cytoskeletal mechanical stress (3) during the microdissection process was negligible. In contrast, the relative HSP72 mRNA content of EX single fibers significantly increased 2 h following 15 min of fatiguing tetanic contractions when normalized to either HSP60 or cardiac α-actin mRNA content (Fig. 3B).

Previous studies have demonstrated increased HSP72 mRNA production in individual cultured myotubes in response to hypoxia (4) and heat shock (40, 65). However, the findings of the present study are the first to demonstrate an elevation of HSP72 mRNA content in individual adult isolated skeletal muscle cells in response to contractions. The findings of the present study are in temporal agreement with Walsh et al. (72) who demonstrated a significant acute elevation of HSP72 mRNA (7.5-fold) only at the 2-h time point following the completion of a single bout of high-intensity exercise in humans. Although the relative linear fold change from rest of HSP72 mRNA (18.5 ± 7.5-fold when normalized to HSP60) in the present study is substantially higher than the acute response previously reported by Walsh et al. (72). Neufler et al. (50a) have reported larger (50-fold) changes in HSP72 mRNA after 24 h following the cessation of exercise. However, the large acute increase in HSP72 mRNA in the present study may be partly a result of large intercell variations in the HSP72 mRNA response, which are amplified in single cell studies. For example, if the relative linear fold change of HSP72 mRNA content from rest of individual fibers in the 2-h group is converted to base 2-log fold change (which minimizes the influence of individual outliers) the result is 2.39 ± 0.8 (normalized to HSP60). When this mean base 2-log fold change value is transformed directly back to linear fold change, the result is a relative linear fold change of 5.2 ± 1.7, a value close to that described by Walsh et al. (72).

Immediate, acute elevations of HSP72 mRNA in response to exercise has also been demonstrated in excised whole muscle (57) and in skeletal muscle biopsied from exercising humans (15, 47, 52, 55, 71) and horses (53). However, debate regarding heat as a mitigating influence in transcriptional activation in these studies has remained (47, 71). The findings of the present study, in which labile heat production was negligible (31), appear to confirm recent findings (48) that factors other than heat exposure, such as alterations in cytosolic [Ca²⁺] (28), decreases in phosphorylation potential (10, 15), or reactive oxygen species production (16), contribute to exercise-induced transcription of HSP72.

**Perspectives and Significance**

The present study describes the development of a novel technique to isolate and quantify mRNA in isolated single skeletal muscle fibers using HSP60 and cardiac α-actin as endogenous normalizing genes, and demonstrated that a single bout of fatiguing contractions is sufficient to elevate HSP72 mRNA, independently of alterations in temperature or signal...
transduction originating from extracellular sources (e.g., cytokine, catecholamine, hormone, etc.). These findings in isolated amphibian myocytes confirm previous findings in mammalian whole muscle, and establish a stable model of HSP72 induction in single cells.

These findings may be pertinent in the design of future therapeutics for skeletal muscle injury and aging. Increased levels of skeletal muscle HSP72 have been shown to be protective and to improve recovery following damage (42, 45), and decreases in HSP72 may be associated with the age-related decline in skeletal muscle function (42). The single skeletal muscle fiber model of contraction-induced transcription developed in the present study permits greater control, specificity, and fidelity of the intracellular factors that regulate the induction of specific genes in skeletal muscle following exercise. This model is unique in that discreet intra- and extracellular factors including cytosolic calcium, extracellular PO2, reactive oxygen species, and muscle fiber types can be individually investigated as potential mechanisms of exercise-induced HSP72 induction.

GRANTS

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