HIGHLIGHTED TOPIC | Molecular Adaptations to Exercise, Heat Acclimation, and Thermotolerance

Cytosolic calcium transients are a determinant of contraction-induced HSP72 transcription in single skeletal muscle fibers

Creed M. Stary1,2 and Michael C. Hogan1

1Department of Medicine, University of California, San Diego, La Jolla, California; and 2Department of Anesthesiology, Perioperative and Pain Medicine, Stanford University School of Medicine, Stanford, California

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Stary CM, Hogan MC. Cytosolic calcium transients are a determinant of contraction-induced HSP72 transcription in single skeletal muscle fibers. J Appl Physiol 120: 1260–1266, 2016. First published February 11, 2016; doi:10.1152/japplphysiol.01060.2015.—The intrinsic activating factors that induce transcription of heat shock protein 72 (HSP72) in skeletal muscle following exercise remain unclear. We hypothesized that the cytosolic Ca2+ transient that occurs with depolarization is a determinant. We utilized intact, single skeletal muscle fibers from Xenopus laevis to test the role of the cytosolic Ca2+ transient and several other exercise-related factors (fatigue, hypoxia, AMP kinase, and cross-bridge cycling) on the activation of HSP72 transcription. HSP72 and HSP60 mRNA levels were assessed with real-time quantitative PCR; cytosolic Ca2+ concentration ([Ca2+]cyt) was assessed with fura-2. Both fatiguing and nonfatiguing contractions resulted in a significant increase in HSP72 mRNA. As expected, peak [Ca2+]cyt remained tightly coupled with peak developed tension in contracting fibers. Pretreatment with N-benzyl-p-toluene sulfonamide (BTS) resulted in depressed peak developed tension with stimulation, while peak [Ca2+]cyt remained largely unchanged from control values. Despite excitation-contraction uncoupling, BTS-treated fibers displayed a significant increase in HSP72 mRNA. Treatment of fibers with hypoxia (PO2: <3 mmHg) or AMP kinase activation had no effect on HSP72 mRNA levels. These results suggest that the intermittent cytosolic Ca2+ transient that occurs with skeletal muscle depolarization provides a sufficient activating stimulus for HSP72 transcription. Metabolic or mechanical factors associated with fatigue development and cross-bridge cycling likely play a more limited role.

HSP70; exercise; fatigue; RTq-PCR; gene; ATP; O2; Ca2+

NEW & NOTEWORTHY

The activating factors for contraction-induced HSP72 transcription in skeletal muscle remain unclear. The present study utilizes single skeletal muscle fibers to demonstrate that the intermittent cytosolic Ca2+ transient that occurs with skeletal muscle depolarization provides a sufficient stimulus to activate HSP72 transcription. Metabolic or mechanical factors associated with fatigue and cross-bridge cycling likely play a more limited role.

HEAT SHOCK PROTEIN 72 (HSP72/HSP70-1) is an inducible member of the HSP70 family of cytoprotective chaperones. Cell stress triggers the activation of constitutively expressed HSP72 to rapidly provide protection without necessitating de novo protein synthesis, while transcriptional activation provides a sustained protective response. Experimentally upregulated HSP72 transcription has been shown to reduce organ injury in a variety of animal models (16, 25, 41). Specific to skeletal muscle, HSP72 has been shown to play a role in minimizing injury severity following freeze injury (36), slow the age-related decline in skeletal muscle function (34) and disuse atrophy (8), delay the progression of muscular dystrophy (14), promote skeletal muscle growth (33), and augment mitochondrial biogenesis (18).

Exercise is a nonspecific stress that activates HSP72 transcription resulting in elevations in both intracellular and circulating levels of HSP72 (19), thereby promoting both local and remote cytoprotective effects. However, the intracellular mechanisms responsible for exercise-induced transcriptional activation of HSP72 remain undetermined. Skeletal muscle excitation-contraction (E-C) coupling is associated with several potential stimuli, including the generation of heat (44), mechanical stress secondary to cross-bridge cycling (3), activation of AMP kinase (24), decreases in intracellular PO2 (21), and rapid release and resequstration of Ca2+ from the sarcoplasmic reticulum (SR) to cytosol (i.e. Ca2+ “transients”). High-intensity/fatiguing contractions are associated with additional potential stimuli for HSP72 transcription secondary to severe disruptions in intracellular homeostasis, including generation of oxidants (43), accumulation of metabolic byproducts (58), alterations in the phosphorylation potential (23, 38), and disruptions in intracellular Ca2+ handling (59). Human and in vivo animal studies demonstrate an increase in skeletal muscle HSP72 activity in response to both high- and low-intensity exercise (for review, see Ref. 37). However, fiber type and motor unit recruitment heterogeneity in these models impair the ability to determine the exercise intensity at the cellular level, and it remains unknown whether skeletal muscle fatigue is a necessary component for HSP72 activation. Moreover, determination of potential intracellular transcriptional signals in these models is complicated by alterations in intracellular substrate availability, extracellular pH, metabolic waste product removal, and the potential for other extracellular signaling sources.

Unlike whole animal and whole muscle models, in the isolated intact single skeletal muscle fiber model substrate availability, waste removal, and temperature are predominantly determined by the extracellular environment (30), which can
be easily set and maintained. We have previously employed the intact single skeletal muscle model to measure the time course of activation of HSP72 transcription in individual single fibers following fatiguing contractions (49). By virtue of being a single cell model, fiber type and motor unit recruitment heterogeneity are removed; as is the potential for extracellular-sourced HSP72 activating signals (26). In the present study we utilized this model to investigate several potential determinants of contraction-induced activation of HSP72 transcription in skeletal muscle. In particular we tested 1) whether the development of fatigue and/or cross-bridge cycling were necessary to activate HSP72 transcription; and 2) whether the intermittent intracellular Ca^{2+} transient associated with skeletal muscle contraction was sufficient to activate HSP72 transcription.

**MATERIALS AND METHODS**

*Animals.* Female adult *Xenopus laevis* were used in these studies. All methods involving animals were approved by the University of California-San Diego Animal Use and Care Committee and conformed to National Institutes of Health standards.

*Experimental design.* We utilized intact, single skeletal muscle fibers from *Xenopus laevis* to test the role of cytosolic Ca^{2+} and several exercise-related factors (fatigue, hypoxia, AMP kinase, and cross-bridge cycling) on the activation of HSP72 transcription. HSP72 mRNA levels in single fibers at rest were compared with levels in fibers following 1) electrical stimulation at fatiguing or nonfatiguing frequencies; 2) hypoxia; 3) activation of AMP kinase; or 4) electrical stimulation following E-C uncoupling with N-benzyl-p-toluene sulfonamide (BTS).

*Single skeletal muscle fiber model.* Single skeletal muscle fibers were isolated and prepared as described previously (20). Briefly, frogs were doubly pithed and the lumbrical muscles (II-IV) were removed. To minimize size and fiber-type heterogeneity, individual, living skeletal muscle fibers of a single type (fatigue-sensitive, glycolytic) were identified and isolated, as previously described (48). Single muscle fibers were dissected with tendons intact in a chamber perfused with physiological Ringer’s solution at a pH 7.0 and platinum clips were attached to the tendons. Muscle fibers were then mounted in a Ringer’s solution-filled glass chamber, and placed on the stage of an inverted microscope equipped for epi-illumination. One tendon was fixed and the contralateral was attached to an adjustable force transducer (model 400A; Aurora Scientific, Aurora, Ontario, Canada), allowing the muscle to be set at optimum sarcomere length. The analog signal from the force transducer was recorded with a digital data acquisition system (AcqKnowledge v3.8; Biopac Systems, Santa Barbara, CA). In this single cell model, the minimal amount of internal labile heat produced from contractions is rapidly dissipated via conduction and convection in a well-stirred medium (30). Therefore, individual muscle fibers were perfused throughout the experiment to maintain a stable temperature (22°C) and to reduce the occurrence of an appreciable unstirred layer surrounding the cell. Tetanic contractions were elicited using direct (8-10 V) electrical stimulation following E-C uncoupling with carboxamide-1-b-riboside-Z-riboside (AICAR; Calbiochem, La Jolla, CA; n = 6) for 15 min or low Po2 (<3 mmHg) Ringer’s solution (n = 7), respectively. Low Po2 Ringer’s was generated via equilibration with 3.5% CO2, balance N2, in a temperature controlled flask and perfused at 1 ml/min with 20-ml glass syringes and an automated syringe pump (model no. sp100i; World Precision Instruments, Sarasota, FL). In-chamber Po2 was monitored with a precalibrated Clark-style Po2 electrode with internal reference (Aurora Scientific, Aurora, Ontario, Canada), with the output signal collected on the data acquisition system.

*Relative cytosolic Ca^{2+} concentration measurements.* Single muscle fibers were microinjected with the cell-impermeable cytosolic Ca^{2+} concentration ([Ca^{2+}]_{cyt}) indicator fura-2 (F-1200; Invitrogen, Carlsbad, CA) immediately following dissection. The [Ca^{2+}]_{cyt}-dependent signal was assessed with an epifluorescent microscope system consisting of a Nikon inverted microscope with a ×40 fluor light objective and a DeltaScan illumination and detection system (Photon Technology International, South Brunswick, NJ) as described previously (47). Fura-2-injected fibers were illuminated sequentially (20 Hz) with two excitation wavelengths of 340 and 380 nm, and the resulting fluorescence emission was measured at 510 nm. The ratio of 340/380-nm fluorescence was used to obtain the relative changes in [Ca^{2+}]_{cyt} (47).

*RNA extraction and cDNA synthesis.* In all experimental protocols single fibers were allowed to recover for 2 h before isolation of total cellular RNA, a time point we have previously demonstrated to coincide with the peak of postcontraction HSP72 mRNA levels in these single muscle fibers (49). Isolation of total cellular RNA from individual muscle fibers was performed as previously described (49). Briefly, individual muscle fibers were manually cleaned of residual cellular debris and removed from the tendon. Single fibers were then introduced into 0.5 ml of TRZol reagent and RNA was precipitated and applied to a column supplied in the Micro RNeasy Total RNA isolation kit (Qiagen). Washing of the column, DNase treatment, and elution of total RNA in 14 µl of DEPC-treated water were performed according to the manufacturer’s instructions. First-strand cDNA synthesis was performed on the total amount of RNA using a ThermoScript Taq-free kit (Invitrogen) per the manufacturer’s instructions.

*Real-time quantitative polymerase chain reaction.* We have previously employed TaqMan real-time quantitative polymerase chain reaction (RT-qPCR) assays in analysis of transcriptional activation in isolated single skeletal muscle fibers (49). Sequence-specific TaqMan-MGB primers and probes were designed to be specific to *Xenopus laevis* gene sequences recorded in GenBank for HSP72/HSP70-1 (GenBank accession no. BC078115) and HSP60 (GenBank accession no. BC041192) as previously described (49). RT-qPCR primer sequences were as follows: HSP72 forward: TGTAGTGCAGTGTGACTT-ATGTTTCTT; and HSP72 reverse: TCTCTCTTATACTCCACTTGTCCATT; and HSP60 forward: GATGCTGTGGCTGTGACAATG; and HSP60 reverse: ACTTCCCCAGCTTTGTTCGA. 6-FAM conjugated RT-qPCR detection sequences were as follows: HSP72: TC-CAAGTGGTGAGCGAT; and HSP60: CCCAAGGGAAGAAC.

RT-qPCR was performed as previously described (49) on an MX3000P system (Stratagene, La Jolla, CA) using TaqMan Master Mix (Invitrogen) with internal ROX reference dye (Stratagene) according to the manufacturer’s instructions. In addition, no-template and no-reverse transcription control reactions were included to test for...
contaminating genomic DNA in the RNA sample and/or primer-dimer amplification. The threshold cycle (Ct) of each reaction was calculated with MX-Pro software v3.0 (Stratagene). For each sample of RNA tested, duplicate Cts were obtained and averaged. The fold change of HSP72 mRNA of individual muscle fibers was determined by the relative quantification method (ΔCt) as described previously (49), and as originally reported by Livak and Schmittgen (32). The fold change of HSP72 of single muscle fibers in the contraction treatment group was analyzed with HSP60 as the internal reference (housekeeping) gene. We have previously determined that HSP60 mRNA levels remain unchanged following contractions in these single skeletal muscle fibers (49).

Data and statistical analysis. All data are reported as means ± SD. Changes between groups over time were tested by mixed-model two-way ANOVA for two independent groups with repeated measures. Relative fold changes in RT-qPCR amplicon content and single fiber cross-sectional area between treatment groups were tested by two-way ANOVA for two independent groups with repeated measures. Changes between groups over time were tested by mixed-model one-way ANOVA. When significant F values were present, a Bonferroni post hoc test was employed for determination of between-group differences. Statistical significance was accepted at P < 0.05.

RESULTS

The mean diameter of all fibers was 90.5 ± 4.1 μm, consistent with fast-twitch, glycolytic fiber type (48, 54), with no difference between groups.

The development of fatigue is not necessary for contraction-induced activation of skeletal muscle HSP72 transcription. In fibers subjected to 15 min of low-frequency (0.1 Hz) electrical stimulation, peak developed tension did not decrease throughout the stimulation period (Fig. 1A, top, and B). However, in fibers subjected to high-frequency (0.33 Hz) electrical stimulation, peak developed tension decreased significantly (to 12 ± 6% of initial peak developed tension) by the end of the stimulation protocol (Fig. 1A, bottom, and E), representing the development of fatigue. As previously observed (49), fatiguing contractions resulted in a significant increase in HSP72 mRNA levels (Fig. 1C). However, a comparable increase in HSP72 was also observed in the low frequency (nonfatiguing) stimulation protocol.

Intermittent intracellular Ca2+ transients in the absence of cross-bridge cycling are sufficient to activate HSP72 transcription in single skeletal muscle fibers. Force tracings during 15-min 0.33-Hz contractions for representative individual control and BTS muscle fibers are illustrated in Fig. 2A, top and bottom, respectively. Incubation with BTS resulted in a significant (P < 0.05) reduction in developed peak tension to 8 ± 3% of pre-BTS values, which was maintained throughout the stimulation protocol in all muscle fibers (Fig. 2A, A and B). As expected, relative peak [Ca2+]cyr of control muscle fibers decreased in concordance with reduced peak developed tension throughout electrical stimulation (Fig. 2C, top). However, in BTS-treated muscle fibers mean relative peak Ca2+ fluorescence of BTS muscle fibers was maintained (Fig. 2, C and D) despite impairment in developed tension, indicating uncoupling of the Ca2+ transient from cross-bridge cycling. Despite E-C uncoupling, electrical stimulation following BTS incubation resulted in an increase in HSP72 mRNA to levels similar to electrical stimulation under control conditions (Fig. 2E).

Finally, in the absence of electrical stimulation no change in HSP72 mRNA was observed with BTS treatment, hypoxic

Fig. 1. Contraction-induced heat shock protein 72 (HSP72) transcriptional activation occurs independently of fatigue development. A: individual tracings of developed tension in single skeletal muscle fibers stimulated to contract at either low frequency (0.1 Hz, top) or high (0.33 Hz, bottom). B: high-frequency (0.33 Hz) electrical stimulation results in a significant decrease in peak developed tension, described as the development of fatigue; a.u., arbitrary units. C: both low-intensity (nonfatiguing) and high-intensity (fatiguing) contractions result in a significant increase in HSP72 mRNA levels. Data are means ± SD. *Significant (P < 0.05) difference from time-matched control.
exposure, or incubation with 2 mM AICAR [a concentration previously demonstrated (11) to activate AMP kinase in skeletal muscle cell culture; Fig. 2E].

**DISCUSSION**

The HSP70 family plays a critical role in maintaining the stability of protein folding and protein-protein interactions and promotes cell survival during stress by inhibiting protein aggregation. Previous investigations in whole animal and human models have demonstrated that a single bout of exercise can induce an elevation in HSP72 mRNA (10, 40, 42, 44, 52). We have previously shown in the intact, isolated single skeletal muscle fiber model that intracellular signals associated with a single bout of high-intensity (fatiguing) contractions are sufficient for activation of HSP72 transcription (49), independent of the generation of heat. This observation agrees with previous experiments utilizing thermal clamps (46, 60) suggesting that exercise-related factors other than temperature serve to activate HSP72 transcription but is at odds with studies suggesting a minimum temperature threshold for HSP72 activation (2, 15). This discrepancy may in part be explained by potential differences in HSP72 activation between species or possibly by loss of heat-modulated extracellular signals in whole muscle and animal studies not present in the single fiber model.

By utilizing the single fiber model, the results from the present study are the first to demonstrate that activation of skeletal muscle HSP72 transcription following contractions is not dependent on the development of fatigue and the associated disruption in metabolic homeostasis. Furthermore, by dissociating free Ca\textsuperscript{2+} from contractions in these single fibers, this study is the first to demonstrate that activation of HSP72 transcription can result from physiologic changes in free Ca\textsuperscript{2+}, independently of heat generation and/or cytoskeletal mechanical stress (Fig. 3). These results suggest that targeted

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Fig. 2. Effect of excitation-contraction (E-C) uncoupling. A: tracings of developed tension from control (top) and N-benzyl-p-toluene sulfonamide (BTS)-treated (bottom) single skeletal muscle fibers. B: treatment with BTS resulted in significant impairment of cross-bridge cycling, resulting in reduction in the development of tension throughout the stimulation time-course. C: fura-2 fluorescence (representing changes in [Ca\textsuperscript{2+}]\textsubscript{cyt}) in control (top) and BTS treated (bottom) single fibers demonstrate preservation of cytosolic Ca\textsuperscript{2+} cycling with electrical stimulation, which persisted throughout the stimulation time course (D). E: despite uncoupling of Ca\textsuperscript{2+} from cross-bridge cycling with BTS, electrical stimulation resulted in an increase HSP72 mRNA levels comparable to control fibers. In the absence of electrical stimulation, treatment of single fibers with BTS, low PO\textsubscript{2}, or the AMP-kinase activator 5-aminoimidazole-4-carboxamide-1-b-riboside-Z-riboside (AICAR) did not result in any change in HSP72 mRNA levels comparable to control fibers. Data are means ± SD. *Significant (P < 0.05) difference from time-matched control.
pharmacologic therapies that augment or mimic skeletal muscle intracellular Ca\textsuperscript{2+} cycling may provide a pathway to enhance and sustain transcriptional activation of this cytoprotective protein.

The transient release and resequestration of Ca\textsuperscript{2+} from the SR is a fundamental component of skeletal muscle E-C coupling. In skeletal muscle, free cytosolic Ca\textsuperscript{2+} has been shown to play an important role in regulating oxidative enzyme expression, mitochondrial biogenesis, and expression of fiber-type-specific proteins (6). Jorquera et al. (27) have previously demonstrated that depolarization of cultured rat myotubes leads to increased transcriptional activity of the HSP70 family of chaperones, an effect blocked by chelation of cytosolic Ca\textsuperscript{2+} (7) and O2 availability (4, 51) has been shown to regulate the phosphorylation potential likely occurred during electrical stimulation secondary to Ca\textsuperscript{2+} cycling. While the phosphorylation potential was not directly assessed in this study, we utilized two indirect approaches to test whether pathways mediated by alterations in phosphorylation potential can activate HSP72 transcription in skeletal muscle: by reducing O2 availability and by activating AMP kinase. We have previously demonstrated in these single muscle fibers that a reduction in O2 availability has the capacity to disrupt the phosphorylation potential significantly (28, 29, 31, 35, 52). These observations suggest that the development of fatigue (and the associated intracellular metabolic disruptions that occur during fatigue) are not necessary to activate HSP72 transcription in these skeletal muscle fibers. Motor unit recruitment heterogeneity may be responsible for previous findings in whole animal and human exercise models that suggest a relationship between work intensity and HSP72 activation. The results from the present study instead suggest that alternative, fatigue-independent, stimuli that occur coincident with skeletal muscle contractions (such as Ca\textsuperscript{2+} transients) are sufficient to activate HSP72 transcription.

Although the development of fatigue was not necessary to activate HSP72 transcription in the skeletal muscle fibers used in the present study, we have previously demonstrated that SR Ca\textsuperscript{2+} cycling may account for up to 40% of the total metabolic cost of contractions (55). Therefore, despite inhibition of cross-bridge cycling with BTS, at least minimal alterations in phosphorylation potential likely occurred during electrical stimulation secondary to Ca\textsuperscript{2+} cycling. While the phosphorylation potential was not directly assessed in this study, we utilized two indirect approaches to test whether pathways mediated by alterations in phosphorylation potential can activate HSP72 transcription in skeletal muscle: by reducing O2 availability and by activating AMP kinase. We have previously demonstrated in these single muscle fibers that a reduction in O2 availability has the capacity to disrupt the phosphorylation potential (22). Alterations in both the phosphorylation potential (7) and O2 availability (4, 51) have been shown to regulate transcriptional activation in skeletal muscle. While SR Ca\textsuperscript{2+} cycling may generate alterations in the phosphorylation potential, in the present study neither a reduction in O2 availability nor chemical activation of AMP kinase resulted in any change in HSP72 mRNA levels, suggesting that pathways mediated by alterations in the phosphorylation potential have a limited effect on HSP72 transcriptional activation in these single fibers. These findings are supported by a recent study by...
Egawa et al. (9), who observed a reduction in HSP72 with AMP-kinase activation in skeletal muscle myotubes.

Another possible mechanism contributing to contraction-induced activation of HSP72 transcription is production of reactive oxygen species (ROS). Alterations in cellular REDOX state have been observed in skeletal muscle with electrical stimulation despite E-C uncoupling with BTS (45), and induction of HSP72 in skeletal muscle by ROS has been proposed following the observation of an attenuated HSP72 response to exercise with antioxidant pretreatment (12). However, as hypoxia is a principle trigger for generation of ROS in skeletal muscle (61), a decrease in O2 availability would be predicted to activate HSP72 transcription, which was not observed in these single fibers. This observation is supported by negative follow-up studies investigating the effect of antioxidant supplementation with exercise induction of HSP72 (17, 39) and the observation of exercise-induced markers of oxidative stress in skeletal muscle in the absence of changes in HSP72 levels (1). Future studies investigating the direct effect of elevated levels of ROS on activation of skeletal muscle HSP72 transcription independent of contractions would serve to clarify the potential role ROS may play.

Finally, the results of the present study were restricted to measurements of HSP72 mRNA in skeletal muscle of a single fiber type (fast-twitch, glycolytic). Future studies assessing the translational and transcriptional response in alternative fiber types will help to verify the translational relevance of the findings in the present study to whole muscle/animal models. However, Carmeli et al. (5) demonstrated that exercise-induced activation HSP72 transcription occurred independently of fiber type in the rat hindlimb. Furthermore, although posttranscriptional modulation (such as translational silencing by microRNAs) can uncouple transcription from protein expression, a comparable degree of increase in HSP72 mRNA and protein levels was observed by Carmeli et al. (5), suggesting a high degree of transcriptional/translational coupling. However, future investigations determining activation of constitutively expressed HSP72 and/or posttranscriptional regulation of HSP72 mRNA may yield further insight into regulatory control of HSP72 induction and the clinical therapeutic potential of skeletal muscle HSP72 modulators.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.M.S. conception and design of research; C.M.S. performed experiments; C.M.S. analyzed data; C.M.S. and M.C.H. interpreted results of experiments; C.M.S. prepared figures; C.M.S. drafted manuscript; C.M.S. and M.C.H. edited and revised manuscript; C.M.S. and M.C.H. approved final version of manuscript.

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