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

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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 resequenching 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 intact single skeletal muscle model to measure the time course of activation of HSP72 transcription, HSP72 mRNA levels were assessed in single fibers (n = 6) pretreated with a 15-min incubation period in Ringer’s solution plus 10 μM of BTS (Sigma Chemicals) before 15 min of electrical stimulation (0.33 Hz). To test whether BTS incubation alone was sufficient to activate HSP72 transcription, fibers (n = 4) were treated with a 15-min incubation in Ringer’s solution with 10 μM BTS, without contractions

Finally, to determine if HSP72 transcription is stimulated by AMP-kinase activation and/or decreased O\(_2\) availability, single fibers were subject to incubation in 2 mM 5-aminomidazole-4-carboxamid-1-b-riboside-Z-riboside (AICAR; Calbiochem, La Jolla, CA; n = 6) for 15 min or low Po\(_2\) (<3 mmHg) Ringer’s solution (n = 7), respectively. Low Po\(_2\) Ringer’s was generated via equilibration with 3.5% CO\(_2\), balance N\(_2\), 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 Po\(_2\) was monitored with a precalibrated Clark-style Po\(_2\) electrode with internal reference (Aurora Scientific, Aurora, Ontario, Canada), with the output signal collected on the data acquisition system.

**RNA and DNA extraction.** Total RNA was isolated from 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 TRIzol reagent and RNA was precipitated with a solution of isopropanol and washed with 70% ethanol. Washed RNA was then purified with a RNeasy mini kit (Qiagen). RNA was eluted in 14 μl of DEPC-treated water 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.

**RT-qPCR detection sequences were as follows:** HSP72 forward: TGGATGCAAGTTGACTCTGGAAGCA; HSP72 reverse: TTTCCTTTATACCTCCATATTGCC; and HSP60 forward: GATGTCTGGCTGTGACAAATG; HSP60 reverse: AACTCCCAAGCCTTTGTCGCA. 6-FAM conjugated RT-qPCR detection sequences were as follows: HSP72: TCCAGGTGAGGCGAT; and HSP60: CCCAAGGGGAAGAC.

**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 background signal.
containing 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 Ct values were obtained and averaged. The fold change of HSP72 mRNA of individual muscle fibers was determined by the relative quantification method (ΔCt) as previously described (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 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 B), 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. 2, A and B). As expected, relative peak [Ca2+]cyt 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...
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^{2+}]_{cyt} 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^{2+}]_{cyt}, 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^{2+}]_{cyt}) in control (top) and BTS treated (bottom) single fibers demonstrate preservation of cytosolic Ca^{2+} cycling with electrical stimulation, which persisted throughout the stimulation time course (D). **E**: despite uncoupling of Ca^{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 PO2, 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 compared to control fibers. Data are means ± SD. *Significant (P < 0.05) difference from time-matched control.
DEPOLARIZATION

Excitation-contraction coupling

CROSS-BRIDGE CYCLING

SR Ca\(^{2+}\) release and reuptake

AMP-kinase activation

\([\text{ADP}+\text{P}]/\text{ATP}\)

\(\text{O}_2\) availability

HSP72 TRANSCRIPTIONAL ACTIVATION

Fig. 3. A model of contraction-induced HSP72 transcription in skeletal muscle. Depolarization induces Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), which results in cross-bridge cycling and the production of force. E-C coupling alters the phosphorylation potential through consumption of ATP and generation of ADP and inorganic phosphate (Pi), which is exacerbated by reductions in \(\text{O}_2\) availability to maintain ATP regeneration through oxidative phosphorylation. Alterations in the phosphorylation potential may either directly or indirectly (via AMP kinase) activate transcription. Uncoupling of SR Ca\(^{2+}\) phosphorylation. Alterations in the phosphorylation potential may either directly or indirectly enhance and sustain transcriptional activation of this cytoprotective protein.

In skeletal muscle, free cytosolic Ca\(^{2+}\) is a fundamental component of skeletal muscle E-C coupling. While Ca\(^{2+}\) cycling was maintained, the findings from the present study demonstrate that the intermittent, transient elevation in [Ca\(^{2+}\)]\(_{\text{cyt}}\) associated with electrical stimulation is sufficient to initiate transcription of HSP72 in these single muscle fibers. It has been previously observed that high-intensity “strength” training induces a greater HSP72 transcriptional response than low-intensity “endurance” regimens (31, 35).

For example, a single bout of high-intensity, fatiguing exercise in whole animal and human models has been shown to induce elevations in local and serum HSP72 levels (10, 35, 52, 53, 56), whereas a single bout of moderate, nonfatiguing exercise did not (28, 29, 31, 35, 52). These observations suggest that the transcriptional response of HSP72 to exercise may be dependent on the development of fatigue, which is associated with severe potential activators of transcription, including severe alterations in phosphorylation potential (23, 38), disruptions in cytosolic Ca\(^{2+}\) handling (57), and increases in metabolic intermediates (H\(^+\), inorganic phosphate, and ADP; Refs. 13, 57).

In the present study, a 15-min period of both high-frequency (fatiguing) and low-frequency (nonfatiguing) electrical stimulation elicited a comparable increase in HSP72 mRNA content, suggesting that the development of fatigue (and the associated intracellular metabolic disruptions that occur during fatigue) is 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\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) 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 \(\text{O}_2\) availability and by activating AMP kinase. We have previously demonstrated in these single muscle fibers that a reduction in \(\text{O}_2\) availability has the capacity to disrupt the phosphorylation potential (22).

Alterations in both the phosphorylation potential (7) and \(\text{O}_2\) availability (4, 51) have been shown to regulate transcriptional activation in skeletal muscle. While SR Ca\(^{2+}\) cycling may generate alterations in the phosphorylation potential, in the present study neither a reduction in \(\text{O}_2\) 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 single 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 on 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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