Influences of temperature, oxidative stress, and phosphorylation on binding of heat shock proteins in skeletal muscle fibers

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Larkins NT, Murphy RM, Lamb GD. Influences of temperature, oxidative stress, and phosphorylation on binding of heat shock proteins in skeletal muscle fibers. Am J Physiol Cell Physiol 303: C654–C665, 2012.—Heat shock proteins (HSPs) help maintain cellular function in stressful situations, but the processes controlling their interactions with target proteins are not well defined. This study examined the binding of HSP72, HSP25, and αB-crystallin in skeletal muscle fibers following various stresses. Rat soleus (SOL) and extensor digitorum longus (EDL) muscles were subjected in vitro to heat stress or strongly fatiguing stimulation. Superficial fibers were “skinned” by microdissection and HSP diffusibility assessed from the extent of washout following 10- to 30 min exposure to a physiological intracellular solution. In fibers from nonstressed (control) SOL muscle, >80% of each HSP is readily diffusible. However, after heating a muscle to 40°C for 30 min, >95% of HSP72 and αB-crystallin becomes tightly bound at nonmembranous myofibrillar sites, whereas HSP25 bound at membranous sites only after heat treatment to ≥44°C. The ratio of reduced to oxidized cytoplasmic glutathione (GSH:GSSG) decreased approximately two- and fourfold after heating muscles to 40° and 45°C, respectively. The reducing agent dithiothreitol reversed HSP72 binding in heated muscles but had no effect on the other HSPs. Intense in vitro stimulation of SOL muscles, sufficient to elicit substantial oxidation-related loss of maximum force and approximately fourfold in vitro stimulation of SOL muscles, sufficient to elicit substantial oxidation-related loss of maximum force and approximately fourfold after heat or other stresses, however, are not well defined. Both HSP72 and αB-crystallin can be phosphorylated at several positions, which is proposed to greatly influence their ability to act as chaperones and to exert protective effects in cells (1, 32, 37), although the latter is somewhat controversial (32). In many cell types the small HSPs exist as large multimeric complexes, and it is frequently claimed that the small HSPs exist as large oligomers and to be predominantly in the fully nonphosphorylated state (20). It is also reported that oxidation can modify the structure and effects of HSPs. HSP25 has a single cysteine residue in its α-crystallin-like domain (31), and S-thiolation of HSP25 regulates its multimeric aggregate size independently of phosphorylation (10, 32). HSP72 has five cysteine residues and can form cross-linked dimers (33), and oxidative modification (specifically, S-glutathionylation) is known to increase its chaperone.

HEAT SHOCK PROTEINS (HSPs) are highly conserved and ubiquitously expressed proteins that act as protein chaperones and in stress situations bind and interact with various proteins to help preserve or restore their function (23, 32). A great deal of work in skeletal muscle has focused on the increase in HSP protein and/or mRNA expression levels occurring in hours to days after various stressful stimuli, such as eccentric exercise (11, 17, 26, 27, 39), oxidative stress (50), heat (4, 35), and glycogen depletion (12). The present study focuses instead on the acute responses of HSPs to stresses, examining the effects of heat, contraction, and oxidative stress on the distribution and binding of three important HSPs present in rat skeletal muscle fibers, namely αB-crystallin, HSP25 (also known as HSP27), and HSP72 (also known as HSP70).

In nonstressed muscle fibers the majority (~80% to 95%) of each of these HSPs is readily diffusible within the cytoplasmic space (2, 16, 20, 21, 26, 39). Following various stress interventions the two small HSPs, HSP25 and αB-crystallin, have been found to bind to a wide variety of different cytoskeletal/myofibrillar proteins (2, 16), including desmin (15, 19, 20, 38), titin (15), actin (28, 43), myosin (24), and possibly also Z-disc proteins. Consistent with this, we have recently shown that in rat SOL muscles heated in vitro to 45°C for 30 min, >95% of the HSP25 and αB-crystallin became tightly bound within the fibers and did not diffuse out even when all internal membranes were dispersed with the detergent Triton X-100 (21). HSP72, in contrast, appears to bind in stress situations primarily to membrane proteins, and in particular has been found to bind to and stabilize the structure and function of the sarco(endo)plasmic reticulum Ca2+-ATPase pump (SERCA1a and SERCA2a) in skeletal and cardiac muscle following heat stress (13, 45). In apparent accord, we have recently reported that the absolute amounts of HSP72 in skeletal muscles are much lower than the density of SERCA pumps [HSP72 content ~1.1 and 4.6 μmol/kg wet weight in rat extensor digitorum longus (EDL) and SOL muscle (21), and total SERCA content ~100 and ~20–35 μmol/kg, respectively (34, 49)], that heat treatment of skeletal muscles to 45°C causes tight binding of >95% of the HSP72 present, and that exposure of such heat-treated fibers to Triton X-100 for 10 min caused diffusional loss of a similar proportion (~50% to 70%) of both the HSP72 and the SERCA (21).

The factors influencing the binding of the various HSPs upon heat or other stresses, however, are not well defined. Both HSP25 and αB-crystallin can be phosphorylated at several positions, which is proposed to greatly influence their ability to act as chaperones and to exert protective effects in cells (1, 32, 37), although the latter is somewhat controversial (32). In many cell types the small HSPs exist as large multimeric complexes, and it is frequently claimed that the small HSPs exist as large oligomers and to be predominantly in the fully nonphosphorylated state (20). It is also reported that oxidation can modify the structure and effects of HSPs. HSP25 has a single cysteine residue in its α-crystallin-like domain (31), and S-thiolation of HSP25 regulates its multimeric aggregate size independently of phosphorylation (10, 32). HSP72 has five cysteine residues and can form cross-linked dimers (33), and oxidative modification (specifically, S-glutathionylation) is known to increase its chaperone.
activity (18). αB-crystallin does not have any cysteine residues (31), but its activity may be modified by oxidation of its other amino acid residues (41). Furthermore, the activity and actions of all of the HSPs may be influenced by oxidation of their potential target proteins (5, 40, 42, 44, 50).

As mentioned, HSPs bind within skeletal muscle fibers following eccentric contractions or muscle heating, as is also the case in cardiac muscle following ischemia-reperfusion (9). With each of these stresses there are increases in the intracellular levels of oxidants (and likely also Ca^{2+} ions) (10, 26, 46, 47), which raises the question of whether oxidation directly or indirectly plays a major role in the HSP binding occurring with these stresses. We hypothesized that the cytoplasmic oxidative status is a major determinant of the binding of some or all of the HSPs in skeletal muscle, or at least would correlate with such binding. We investigated this by subjecting rat muscles in vitro to various heat stresses, or to repeated isometric stimulation under conditions known to cause a substantial oxidative-induced reduction in maximum force production (8). We then gauged the level of HSP binding from the extent of diffusional loss occurring in skinned fibers from those muscles, comparing this with a measure of the oxidative status of the cytoplasm (the ratio of reduced to oxidized glutathione, GSH: GSSG). We also examined the effect of the strong cysteine reducing agent, dithiothreitol (DTT), on the binding of the HSPs and the ability of applied exogenous HSP72 to bind at sites in skinned fibers from stressed and nonstressed muscles. The findings indicate that a large increase in cellular oxidation is not sufficient in itself to cause binding of any of the HSPs, but that cysteine oxidation does play a role in binding of HSP72 in at least some circumstances. Furthermore, binding can be brought about by effects of the stress on the binding target(s) rather than on the HSP72 itself.

MATERIALS AND METHODS

Materials and antibodies. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. Antibodies used were against HSP25 (1:2,000 rabbit polyclonal, SPA-801; Stressgen), phosphorylated HSP25 Serine 85 (pHSP25-Ser85; 1:200 mouse monoclonal, ALX-804 – (St. Louis, MO) unless otherwise stated. Antibodies used were against the HSP72 itself.

Whole muscle in vitro stimulation. Rat SOL muscles were stimulated in vitro in the same manner as described recently (8). The isolated SOL muscle was attached between a glass hook and a force transducer in a bath filled with Krebs-Ringer solution (KRS) containing (in mM) 122 NaCl, 2.8 KCl, 1.2 MgSO_4, 1.2 KH_2PO_4, 25 NaHCO_3, with CaCl_2(1.3 mM) and D-Glucose (5 mM) added on the day of the experiment (pH 7.4). The solution was bubbled with 95% O_2 and 5% CO_2 at 30°C for at least 30 min before the start of the stimulation. After 15 min of equilibration, the muscle was electrically stimulated to contract isometrically. Muscle length and stimulation voltage were varied to give maximum twitch force, and the force-frequency characteristics were examined with ~5 tetanic contractions at various frequencies (5–50 Hz; all with pulse duration 1 ms, amplitude 30 V). Force responses were recorded on a chart recorder and personal computer (Powerlab and Chart 5 software; ADInstruments, Australia). Each SOL muscle was then subjected to a prolonged train of tetani, elicited by 1-ms pulses at 50 Hz for 500 ms every 2 s until the peak tetanic force dropped to less than 20% of its initial level (on average muscles were stimulated for ~5 min). To assess whether the maximum tetanic force had recovered, after a 2-min rest the muscle was subjected to a single prolonged 50-Hz test stimulus until the force reached a plateau. The muscle was then immediately removed from the bath and pinned under paraffin oil for skinned fiber segment collection or snap frozen in liquid nitrogen and stored at ~80°C for glutathione analysis.

Whole muscle heat treatment. Intact SOL muscles were pinned in a dish at resting length in paraffin oil or in bubbled KRS preheated to the desired temperature (37°C, 40°C, 44°C, or 45°C). The dish was placed in a water bath at the required temperature for 30 min. After treatment, the muscle was placed in a fresh dish with paraffin oil at room temperature (RT) and slowly cooled and maintained at ~10°C for fiber collection, or blotted on filter paper and snap frozen in liquid nitrogen for glutathione analysis. The data for the 45°C heat treatment have been presented previously (21) but was collected in the same series of experiments as the other temperature data here.

Western blotting for protein diffusibility and quantification. As described previously (25), total protein samples were separated on Criterion Stain Free gels (Bio-Rad, Hercules, CA) (30) or in some cases on 12% SDS polyacrylamide gels. Proteins were then transferred from the gel onto nitrocellulose membrane and blocked with blocking buffer (5% skim milk in Tris-buffered saline with Tween 20) for 2 h. After proteins were blocked, membranes were generally cut at ~55 kDa into two sections and the sections were probed with the required primary antibody overnight at 4°C and for 2 h at RT, constantly rocked. Subsequent to washes, secondary antibodies were added.
muscle were mechanically skinned and then placed individually in muscles were either heated to 45°C for 30 min (see whole muscle heat vortexed briefly (2 s) before removal from any wash solution. Fibers before being wiped clean after every immersion. All fibers were with exogenous HSP72, they were washed in double distilled water derived simply from the density of the specific immunoreactive band in the W lane expressed relative to the sum of the specific bands in the fiber segment, and the fiber wash (W) sample contained all the components that had been freely diffusible within the fiber. There-fore, for a given protein the percentage diffusible within the fiber was derived simply from the density of the specific immunoreactive band in the W lane expressed relative to the sum of the specific bands in the W and matching F lanes.

Addition of exogenous HSP72 to heat-treated fibers. EDL and SOL muscles were either heated to 45°C for 30 min (see whole muscle heat treatment) or not heated. Segments of fibers dissected from each muscle were mechanically skinned and then placed individually in 5 μl of Buffer A for 10 min (denoted as the initial wash solution, WI). After this either the fiber was washed as per normal (see above), or the wash alone was kept and the fiber was placed for 10 min in Buffer A containing 1.3 μmol purified HSP72 per liter. In the latter case, the fiber was subsequently briefly immersed (for ~10 s) in a large volume (~500 μl) of Buffer A to remove any excess HSP72, and then washed for 20 min in another 5 μl of Buffer A, and then finally collected in 5 μl of Buffer A for analysis alongside its corresponding first wash (WI). To ensure the forceps did not become contaminated with exogenous HSP72, they were washed in double distilled water before being wiped clean after every immersion. All fibers were vortexed briefly (~2 s) before removal from any wash solution. Fibers and all matched wash solutions were kept at ~20°C until analyzed by Western blotting. In addition to the fiber sample set, each Western blot had a sample of the same SOL homogenate on each gel to cross-

Glutathione assay. Intact muscles were frozen in liquid nitrogen following dissection or first given a stress treatment [straight out of animal (control), stimulated, 40°C heated and 45°C heated] and were subsequently prepared for GSSG and total glutathione measurements as per manufacturer’s instructions (Cayman Glutathione assay Catalog No. 703002, Ann Arbor, MI) and as described previously (8). Briefly, muscles were weighed and homogenized immediately (1:10: mass:volume) in cold 50 mM MES buffer (supplied by manufacturer, Caymen Glutathione assay) before being centrifuged at 10,000 g for 15 min at 4°C after which the supernatant was collected. Supernatants were deproteinized using 1.25 M metaphosphoric acid and left for 5 min at RT, after which time samples were centrifuged at RT at 2,000 g for 2 min. The supernatant was removed and stored at ~20°C until analyzed for glutathione. On the day of the assay, all samples were vortexed with TEAM reagent (4 M triethanolamine) and 2-vinylpyridine (1 M 2-vinylpyridine) was added to GSSG specific samples. Duplicate standard curves of both GSSG and GSH were loaded onto each plate, along with triplicates of each sample. Each well was loaded with assay cocktail (according to manufacturer’s instructions). The absorbance of each well was measured at 5-min intervals for 30 min at 405–414 nm using a RA Anthos Reader 2010 (Biochrom, Cambridge, UK) and ADAP Plus software (Biochrom). GSSG and total GSH amounts were calculated by comparing sample values against the standard curve.

Statistics. Data are expressed as means ± SE, with the number of samples being the number of individual fibers studied, and the number of muscles shown are in brackets. Statistical significance was examined using Student’s t-tests (paired or unpaired as appropriate). In cases where data were not normally distributed, significance was tested using the nonparametric Mann Whitney unpaired rank test; in the case of multiple variables, significance was tested using one-way ANOVAs. A probability value (P) <0.05 was deemed as significant.

All statistical analyses and data fits were performed using GraphPad Prism version 4.

RESULTS

Diffusibility of HSPs in fibers from heat-stressed muscle. We reported recently that when SOL muscles are heated in vitro at 45°C for 30 min the majority (~95%) of each of the three HSPs becomes tightly bound within the fibers (21), with αB-crystallin and HSP25 binding to cytoskeletal/contractile proteins and HSP72 binding to membrane-linked sites, likely in large part to the SERCA (see INTRODUCTION). A further major difference between the binding of the HSP72 and the small HSPs is also apparent in muscles subjected to less stringent heat treatments (e.g., Fig. 1). Soleus muscles were heated in vitro for 30 min in paraffin oil at various temperatures (37°C, 40°C, 44°C, or 45°C) or in KRS bubbled with 95% O2 at 37°C. As described previously, segments of individual fibers were skinned by microdissection under paraffin oil and then both a set period (10 or 30 min) in a physiological wash solution that broadly mimicked the normal cytosol (see MATERIALS AND METHODS) to determine the proportions of the HSPs diffusing into the wash solution (W) or remaining in the skinned fiber (F). Control fibers from the contralateral (nonheated) muscle in each experiment were similarly skinned and washed, with collection of fibers from the heated and the control muscles interspersed. Each fiber sample (F) was then run on SDS-PAGE with the corresponding wash sample (W) in the adjacent lane to assess the protein constituents of each. Figure 1 is a representative Western blot of the HSPs found in four fiber and wash sets from a SOL muscle heated to 40°C and one set from

Fig. 1. Heat shock protein (HSP25 and αB-crystallin bind in fibers of soleus (SOL) muscle heated at 40°C, but HSP72 remains diffusible. Western blots of HSP72, HSP25, and αB-crystallin remaining in skinned fiber segment (F) or diffusing into wash solution (W) for 4 fiber/wash sets from a SOL muscle heated at 40°C for 30 min (alternating 10- and 30-min wash times) are shown. Left: a fiber/wash set (10-min wash time) and an intact (nonskinned) fiber segment (I) from the nonheated contralateral muscle. Coomassie-stained myosin heavy chain (MHC) and actin reprobe indicate relative amount of muscle tissue loaded in each lane and confirm absence of appreciable myofibrillar contamination in wash solutions.

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the corresponding control muscle. As expected, there was minimal loss of actin or myosin to the wash solution in each case. The most striking feature is that in all four fibers from the 40°C-treated muscle virtually all of the HSP72 diffused out into the wash solution (W), whereas virtually all the HSP25 and the αB-crystallin remained in the fiber (F) in those same cases.

The mean data of the percentage of each HSP remaining diffusible following the various temperature treatments are shown in Fig. 2; the data for the 10- and 30-min diffusion times were not significantly different and are grouped together in the summed results presented. As reported previously (21), in control fibers the majority of each HSP is diffusible and is readily lost to the wash solution. Neither of the 30-min treatments at 37°C (i.e., in vitro immersion in paraffin oil or in KRS bubbled with O2) detectably altered the diffusibility of any of the three HSPs in SOL muscle (Fig. 2). However, heat treatment at 40°C greatly reduced the percentage of diffusible αB-crystallin and HSP25 to only 5±2% and 2±1%, respectively, whereas virtually all of the HSP72 in those fibers remained readily diffusible (Fig. 2). HSP72 only displayed marked binding with heat treatments to ~44°C or above (Fig. 2A). As found previously (21), aldolase remained freely diffusible in every fiber examined even with heat treatment to 45°C (e.g., see Fig. 5). It should be noted there was no significant difference between treated and control muscles in the amount present of any of the HSPs (data not shown), demonstrating that there had been no change in protein expression during these acute treatments.

**Diffusibility of HSPs in fibers from stimulation-stressed muscle.** To assess the effect of intense muscle stimulation on HSP binding, isolated whole SOL muscles from rat were bubbled with 95% O2 in KRS at 30°C and subjected to a demanding stimulation protocol (50 Hz stimulation for 0.5 s every 2 s) until the tetanic force dropped to <20% of the initial level (e.g., Fig. 3A). After such stimulation, maximum force to a prolonged 50-Hz stimulus applied 2 min later was only 25±9% of that elicited before the fatiguing stimulation (n = 5 SOL muscles). This particular protocol was used because we have shown recently that such intense in vitro stimulation of rat SOL muscles in these temperature and O2 conditions results in marked reactive oxygen species-mediated dysfunction of the contractile apparatus (8). Individual fiber segments from the stimulated muscles were skinned by microdissection and exposed to a wash solution for a set period (10 or 30 min) as described above. Figure 3B is a representative Western blot of the proteins remaining within the fiber segments (F) or diffusing out into the wash solution (W), and Fig. 3C presents the mean data. It was found that the great majority of each of the HSPs remained diffusible in the cytoplasm even when a muscle had been subjected to this stressful stimulation protocol, with the percentage of HSP lost to the wash solution showing no significant difference between the control and stimulated muscles for any of the HSPs (Mann Whitney unpaired rank test: HSP72, P = 0.4; HSP25, P = 0.6; αB-crystallin, P = 0.3). Glutathione assay. The extent of oxidative stress caused by the various treatments was gauged from the level of oxidation of cytosolic glutathione. This was assessed by measuring the amounts of reduced glutathione (GSH) and oxidized glutathione (GSSG) for each case (see MATERIALS AND METHODS) and deriving the GSH:GSSG ratio (Fig. 4). In the nontreated

![Fig. 2.](http://ajpcell.physiology.org/) Heat treatment differentially affects diffusibility of the HSPs. Data are mean percentage (+SE) of HSP lost to the wash solution for skinned fibers from SOL muscles maintained in vitro at the indicated temperature either under paraffin oil or in Krebs-Ringer solution (KRS) bubbled with 95% O2. Data ascertained using skinned fiber-wash sets are as in Fig. 1. The number of individual fibers per wash sets for each case is shown beneath the bar, with the number of muscles shown in brackets. **A**: values for each HSP; all 3 HSPs were examined in each fiber in the majority of cases. Con, fibers from the untreated contralateral muscles. In 1 case the contralateral muscle pair was used to directly compare effects of 37°C treatment in paraffin oil and in KRS. Both HSP25 and αB-crystallin became predominantly bound in muscles heated to 40°C, whereas HSP72 only showed similar binding in muscles heated to 45°C. **B**: Significant difference in comparison with paired control muscles (P < 0.05, Mann Whitney unpaired rank test).
muscles most of the glutathione was in the reduced form, with the GSH:GSSG ratio being ~50. The heat treatment at 40°C significantly increased the amount of GSSG in the cytosol, decreasing the GSH:GSSG ratio approximately twofold. The heat treatment at 45°C decreased the GSH:GSSG ratio almost fourfold, and the stimulation protocol had a similar effect (Fig. 4).

Effect of DTT on HSP binding. To further investigate the binding properties of the HSPs, in particular the involvement of oxidation, skinned fibers from heated muscles were exposed to a wash solution containing 10 mM DTT, a strong reducing agent of cysteine residues. The presence of DTT did not significantly alter the tight binding of HSP25 or αB-crystallin in fibers from the heat-treated muscles (e.g., Fig. 5A); the percentages diffusing out in the presence of DTT were 2 ± 1% (n = 7 fibers, 3 muscles) and 0.5 ± 0.5% (n = 7, 3) for HSP25 and αB-crystallin, respectively, for fibers from 40°C-treated muscles, and 8 ± 4% (n = 10, 4) and 5 ± 4% (n = 10, 4) for 45°C-treated muscles, none of which were significantly different from those in the absence of DTT (Mann Whitney unpaired rank tests). In contrast, the presence of DTT in the wash solution significantly increased diffusional loss of HSP72 in skinned fibers from 45°C-treated muscle (mean 34 ± 5%) compared with that occurring in the absence of DTT (Fig. 5B). As expected, SERCA remained in the fiber irrespective of the DTT (e.g., Fig. 5A). These results indicate that the binding of

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![Figure 3](image-url)  
**Fig. 3.** Intense in vitro muscle stimulation does not alter HSP diffusibility.  
**A:** decrease in maximum tetanic force after repeated stimulation of isolated SOL muscle (in KRS at 30°C bubbled with 95% O₂). Initial and final responses are tetanic force elicited by continuous 50 Hz stimulation until force peaked. Repeated 50-Hz stimulation applied for 0.5 s every 2 s until force dropped to ~2% of initial. **B:** Western blot showing HSPs and actin in fiber (F) and wash (W) sets for 3 fibers from a stimulated (Stim) SOL muscle and 1 set from the unstimulated contralateral muscle (Con); the majority of all 3 HSP diffused out into the wash solution. Coomassie-stained MHC and actin reprobe indicate minimal myofibrillar contamination in wash lanes (12% SDS-PAGE gel). **C:** mean percentage (+SE) of each HSP found in wash solution for fibers from stimulated and control nonstimulated SOL muscles; the number of fibers examined is shown under bar, with the number of muscles shown in brackets. There was no significant difference between values for stimulated and control muscle fibers for any of the HSPs.

![Figure 4](image-url)  
**Fig. 4.** Extent of glutathione oxidation in SOL muscles subjected to different stress treatments. Mean amount of reduced glutathione (GSH; A), oxidized glutathione (GSSG; B), and the GSH-to-GSSG ratio (C) in muscles subjected to heat treatment at 40°C or 45°C for 30 min or to vigorous stimulation as in Fig. 3 (‘Stim’) (n = 3 for each case) is shown. Nonstimulated contralateral muscle (Con) was examined in 4 cases. Amount of GSSG significantly increased in muscles subjected to either heat treatment or the stimulation protocol, with the GSH-to-GSSG ratio concomitantly decreased. *Significant difference from control case (P < 0.05); #significantly different from 40°C treatment case. Total glutathione content (i.e., sum of GSH + 2 × GSSG) was not significantly different between the various cases.
the HSP72 involved oxidation of cysteine residues in some manner.

Binding of exogenous HSP72 in fibers. Other experiments were conducted to determine whether exposure to additional (i.e., exogenous) HSP72 increased the amount of HSP72 binding within skinned fibers of heat-treated and non-heat-treated muscles. Segments of individual fibers from SOL and EDL muscles heated to 45°C for 30 min, and from the nonheated contralateral muscles, were skinned as usual and bathed in muscles heated to 45°C for 30 min, and from the nonheated (i.e., exogenous) HSP72 increased the amount of HSP72 binding in each fiber segment and in the corresponding initial wash (WI), normalizing values by the density of myosin heavy chain stain free band for the given fiber segment (i.e., the measure of fiber amount) and running a sample of the same SOL homogenate on each gel to cross-compare values obtained from different gels (see MATERIALS AND METHODS).

In accord with the findings earlier in this study and in our previous study (21), ~85% to 95% of the endogenous HSP72 present in the fibers from the nonheated muscles diffused into the initial wash solution (WI), whereas very little (0–10%) did so in fibers from the 45°C-heated muscles; there was no significant difference in this regard between the fibers subsequently exposed or not exposed to the exogenous HSP72. Figure 6 presents the mean values of the total of the HSP72 found in initial wash (WI) and matching fiber segment for the fibers for each experimental case; the values are expressed relative to the average amount of HSP72 present in the untreated control fibers (i.e., fibers from nonheated muscle that were not exposed to exogenous HSP72). As to be expected, the total amount of HSP72 associated with heated fibers not exposed to exogenous HSP72 was indistinguishable from that for the control fibers, for both the SOL and EDL cases. It is noteworthy that after the application and washout of exogenous HSP72, the skinned fibers from the heated SOL muscles showed a significant increase in total HSP72 (~2-fold), whereas the fibers from the nonheated SOL muscle showed no significant change (Fig. 6A). In contrast, the skinned fibers taken from the heated and nonheated EDL muscles showed ~12-fold and approximately fivefold increase, respectively, in total HSP72 content after application and washout of exogenous HSP72 (Fig. 6B). These data indicate that the number of binding sites for HSP72 is greater than the amount of HSP72 present endogenously, particularly in EDL fibers, and provide novel information about the factors needed for HSP72 binding (see DISCUSSION).

Phosphorylation of sHSP. The extent of phosphorylation at Ser85 on HSP25 and at the Ser59 on αB-crystallin was examined in 40°C-heated and control SOL muscle (Fig. 7). Heated and contralateral muscle samples were run on the same gel, together with a cell-lysate positive for phosphorylated HSP25 Ser85, and probed for both total and phosphorylated forms of the HSPs (e.g., Fig. 7A). Relative to the control muscle, the 40°C-heated SOL muscle displayed approximately threefold greater phosphorylation at Ser85 on HSP25 and ~12-fold greater phosphorophylation at Ser59 on αB-crystallin (Fig. 7, B and C). In addition, the absolute level of phosphorylation at Ser85 on HSP25 could be estimated by comparison with the cell-lysate positive control, assuming the latter indicated 100% phosphorylation (e.g., Fig. 7D). The mean data from analysis of four independent samples (each run on a different gel) indicated that the level of phosphorylation at Ser85 on HSP25 was 35 ± 2% and 9 ± 1% in the heated and control muscle samples, respectively. The absolute level of phosphorylation of

Fig. 5. Dithiotreitol (DTT) reverses HSP72 binding in heat-treated fibers. A: Western blots showing HSPs remaining in skinned fiber (F) or diffusing into wash solution (W) when DTT (10 mM) was absent or present in wash solution (2 cases of each shown); skinned fibers obtained from SOL muscle were treated at 45°C for 30 min. HSP25 and αB-crystallin remained in the fiber irrespective of the presence of DTT, whereas HSP72 diffusion out of the fibers was enhanced in the presence of DTT. Aldolase was readily diffusible in all cases. B: mean percentage (+SE) of HSP72 in wash solution after bathing skinned fibers from 45°C-treated SOL muscle for 30 min in wash solution with or without 10 mM DTT. Numbers of fibers (from 4 muscles) is shown under bars; fibers examined with and without DTT were obtained in interspersed order from each heated muscle. *Significantly different from case without DTT (P < 0.05, Mann Whitney unpaired rank test). SERCA, sarco(endo)plasmic reticulum Ca2+ -ATPase.
Ser59 on αB-crystallin could not be estimated as no positive was available.

**Diffusibility of phosphorylated small HSP.** To investigate whether phosphorylation of the small HSPs influenced their diffusibility, skinned muscle fibers from control and heated SOL muscles were placed in wash solution for 10 min, and Western blotting was used to assess the proportion of the phosphorylated and total HSP in the fiber (F) and wash (W) samples (e.g., Fig. 8, A and B). Analysis of such samples showed that the amounts of phosphorylated HSP25 and αB-crystallin (derived as the sum in the respective F and W lanes) for each fiber) were greater in the fibers from heated muscles compared with the control muscles (Fig. 8, C and D), in general accord with the measurements derived using whole muscle homogenates (Fig. 7). The lower ratio values seen in the single fiber data in Fig. 8 likely result from disproportionate effects of small nonlinearities in band densities on the calculated ratio values; as such the ratios derived from the whole muscle homogenates are likely to be the more accurate values. It is noteworthy that it was apparent from inspection of the individual fiber and wash samples (e.g., Fig. 8, A and B) that the phosphorylated form of each HSP was distributed between the fiber and wash in approximately the same proportion as was the HSP as a whole. For example, in the nonheated fibers, in which most of the HSP is diffusible, the majority of the phosphorylated HSPs were found in the wash. Conversely, in the heated samples, where the total amount of phosphorylated HSP was increased, it was apparent that appreciable amounts of the phosphorylated form of the HSPs diffused out of the fibers into the wash and that this proportion was similar to that for the total HSP (e.g., see αB-crystallin in heated fibers in Fig. 8B). This behavior was investigated quantitatively by plotting the percentage of the phosphorylated HSP in the wash versus the percentage of the total HSP in the wash for all the individual fiber and wash sets (Fig. 8, E and F). Such analysis indicated that the phosphorylated form of each HSP was distributed between the fiber and wash in similar proportion as the total HSP. In other words, the diffusibility of a given HSP was not seemingly influenced by whether or not it was phosphorylated.

**DISCUSSION**

The present findings highlight the very substantial differences between the binding of HSP72 and the small HSPs in skeletal muscle fibers. One striking finding was that binding of the sHSPs was triggered over a relatively sharp temperature range between 37° to 40°C (Fig. 2), whereas HSP72 showed similar sharp temperature dependence but at substantially higher temperature (~44° to 45°C). Because increased generation of reactive oxygen species and related compounds is a common feature of temperature and other stresses triggering HSP binding in skeletal muscle fibers (see INTRODUCTION), the present study examined whether HSP binding was correlated with the oxidative state of the cytoplasm. The level of oxidized glutathione (GSSG) in the cytoplasm was significantly increased by the 40°C treatment (~2-fold), and increased even more with the 45°C treatment (to ~4-fold in total; Fig. 4), suggesting it could be a determinant or correlate of HSP binding for one or both classes of the HSPs. However, it was further found that intense in vitro stimulation increased cytoplasmic GSSG levels to at least as great an extent as the 45°C temperature treatment (Fig. 4) but did not lead to significant levels of binding of any of the HSPs (Fig. 3). Thus the oxidative state of the cytoplasm does not appear to be a primary determinant of the binding of any of the HSPs or a reliable correlate.

Further experiments nevertheless indicated that oxidation does indeed play a role in the binding of HSP72, at least in some circumstances. Specifically, it was found that the presence of 10 mM DTT, a strong reducing agent of cysteine bonds, greatly increased the proportion of HSP72 diffusing out of the heat-treated fibers (Fig. 5). This indicates that binding of HSP72 in the heated fibers was in some way dependent on reversible oxidation of one or more cysteine residues, which might involve formation of intramolecular or intermolecular disulphide bonds or sulphenation, S-glutathionylation, or S-nitrosylation of cysteine residues. HSP72 has five cysteine residues and can form cross-linked dimers involving Cys574 (33). Heat shock cognate protein 70, which is closely related to...
HSP72 and contains four of the same five cysteine residues, is known to undergo S-glutathionylation, which increases its efficacy as a chaperone (18). It is also known that in oxidizing conditions HSP72 adopts a different conformation and binds more readily to target proteins (3), including to SERCA (45). Furthermore, HSP72 has been found to form disulphide cross-links to other proteins in cells treated with oxidizing agents (6). Thus HSP72 binding may be increased in oxidizing conditions both by conformational changes in the HSP72 itself and by direct disulphide linkage to targets, though the latter is probably not as generally relevant because it would only pertain to target proteins with a suitably positioned cysteine residue. In any case both of these processes would be reversed by the presence of DTT, consistent with the present observations that DTT treatment decreased the extent of HSP72 binding in heat-treated muscle fibers (Fig. 5).

A previous study showing that oxidative conditions promote HSP72 binding (3) further found that subsequent application of DTT did not reverse the binding, seemingly in contrast with the present observations. However, this difference in findings is likely to be due to the fact that there was no ATP present in assay conditions in those previous studies, whereas the binding of the HSPs was examined here in the presence of physiological levels of ATP. HSP72 is an ATPase that in its ATP-bound state has a low affinity and high exchange rate with target proteins, but such interaction catalyzes the hydrolysis of the ATP to ADP, resulting in a conformational change in the HSP72 that greatly slows its dissociation from the target until

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**Fig. 7.** Heat treatment causes increased phosphorylation of small HSPs. 

A: Western blots of total and phosphorylated forms of HSP25 (at Ser85) and αB-crystallin (at Ser59) in control and 40°C-heated SOL muscle, together with positive samples of pHSP25Ser85 (right lanes). B and C: mean level of phosphorylation at Ser85 on HSP25 and at Ser59 on αB-crystallin in heat-treated SOL muscle relative to nonheated contralateral muscle (4 independent muscle pairs). Phosphorylated HSP band intensity for each sample was first normalized by corresponding HSP density. #Significantly different for control (P < 0.05). D: phosphorylation at Ser85 on HSP25 in control and heated SOL muscle when expressed relative to positive control on same gel (for data in A). Slope of linear regression (constrained through origin) indicates percentage of HSP25 phosphorylated at Ser85 was ~33% and 10% in heated and control muscles, respectively, assuming positive indicates 100% phosphorylation. AU, arbitrary units.
the ADP is displaced by ATP (22, 23, 48). Thus, in the present study, the heat treatment of the muscles possibly produced S-glutathionylation of most of the HSP72, leading to it tightly associating with target proteins within the fibers (probably predominantly SERCA), and this could be subsequently reversed by application of strongly reducing conditions (10 mM DTT) in the presence of ATP. Because HSP72 did not show similar binding in the stimulated muscles (Fig. 3), it would have to be concluded that increasing the [GSSG] in the cytoplasm three- or fourfold was not enough in itself to cause S-glutathionylation of the HSP, which is consistent with the current view that S-glutathionylation is expected to only occur at a much higher GSSG:GSH ratio (7). Instead, the increased level of reactive oxygen species (e.g., superoxide and per-
oxynitrite) produced by the heat treatment (36, 46) may have directly oxidized the HSP72, which then underwent S-glutathionylation by reacting with free GSH in the cytoplasm (7), thereby enhancing the affinity of HSP for its targets.

Alternatively or additionally, the heat treatment may have been particularly effective because it caused oxidation-induced denaturation of the SERCA proteins, exposing hydrophobic regions, which triggered HSP72 binding (45). Support for the latter comes from our additional finding that exogenous HSP72 bound within muscle fibers even though the HSP72 itself had not been subjected to the heat treatment or oxidizing/glutathionylating conditions (Fig. 6). In this case the HSP72 evidently recognized and bound to targets within the fibers because of alterations in target proteins rather than alterations in the HSP72 itself. It is likely that SERCA is the predominant binding site of the HSP72 in the skinned fibers here, given 1) the propensity of SERCA to bind HSP72 (13, 14, 45), 2) the comparatively high absolute number of SERCA proteins present in muscle fibers (~100 and 20–35 μmol/kg wet weight in EDL and SOL muscle, respectively (34, 49)), and 3) the relatively greater additional HSP72 binding occurring in the EDL fibers compared with SOL fibers (Fig. 6) [see also INTRODUCTION and Ref. 21]. It is noteworthy that with the EDL fibers there was an increase in exogenous HSP72 binding even when the fibers had not been subjected to heating (Fig. 6B), possibly owing to alterations in the target proteins occurring over the prolonged course of the exposure and washout of the exogenous HSP72 (>30 min, without any reducing agents present).

In contrast with HSP72, in stress conditions αB-crystallin and HSP25 bind to cytoskeletal and myofibrillar proteins rather than to membrane proteins such as SERCA (see INTRODUCTION). HSP25 contains one cysteine residue, and S-thiolation of this residue aids dissociation of HSP25 from its multimeric to monomeric forms independently of phosphorylation (10). There are no cysteine residues in αB-crystallin, but its activity may be modified by oxidation of its other amino acid residues (41). The present study found no evidence of oxidation playing a role in the binding of the sHSPs. In particular, as with HSP72 there was no measurable increase in sHSP binding in the fibers from the muscles subjected to intense in vitro stimulation (Fig. 3), even though such stimulation increased the GSSG levels fourfold (Fig. 4) and has been previously shown to cause oxidation-related changes in the contractile proteins resulting in marked reduction in force production (8). Furthermore, the presence of the cysteine-reducing agent DTT did not lead to any unbinding and diffusional loss of the sHSPs from the heated muscle fibers (e.g., Fig. 5A). It is nevertheless possible that oxidation did play a role in the binding of the sHSPs, such as by inducing a conformation change in the target proteins or in the HSPs themselves, but any such effects must be poorly reversible by DTT.

The intensive in vitro stimulation protocol used here involved eliciting repeated isometric contractions of the muscles at a comparatively high duty cycle. Such stimulation would have raised intracellular [Ca^{2+}] to considerable levels for many minutes, and even though this was accompanied by an appreciable increase in oxidants (Fig. 4) it still did not lead to binding of any of the HSPs (Fig. 3). This seems consistent with previous work showing that HSP binding does not readily occur with concentric exercise (20, 39), being instead triggered primarily by eccentric exercise. It nevertheless remains unclear what particular aspect of the eccentric exercise causes the sHSP binding. Given that the sHSPs are seen to bind after eccentric exercise not just to isolated areas of damage but instead fairly uniformly to all Z-bands throughout the fibers (20), it would seem that some widespread or diffuse process must trigger the binding. One possibility could be that the considerable stresses occurring during the lengthening contractions cause widespread structural changes in one or more Z-line proteins, revealing hydrophobic regions that in turn facilitate sHSP binding.

Finally, the findings here suggest that there is no simple relationship between phosphorylation and binding of the sHSPs. Virtually all of the HSP25 became bound in the heat-treated muscles even though only a third or less were actually phosphorylated at Ser85 (Fig. 7), similar to what was found previously for αB-crystallin following eccentric contractions (20). Furthermore, the phosphorylated forms of HSP25 and αB-crystallin were found to bind or diffuse out of the skinned fibers in virtually the same proportion as the nonphosphorylated forms (Fig. 8), arguing against any close causal relationship between phosphorylation and binding. This is consistent with findings in cardiac muscle following ischemia-reperfusion, where phosphorylation was found to be neither necessary nor sufficient for translocation and binding of αB-crystallin (9). Although the present findings argue against phosphorylation having a mandatory role in sHSP binding in skeletal muscle fibers, it is quite possible that phosphorylation influences other key properties of the sHSPs such as their ability to effect refolding or repair of target proteins.

In conclusion, the findings indicate that a large (4-fold) increase in cytoplasmic oxidation is not sufficient in itself to cause binding of any of the HSPs in skeletal muscle fibers. Nevertheless, it is apparent that cysteine oxidation does play a role in binding of HSP72 in at least some circumstances and further that HSP72 binding can be brought about by effects of the stress on the binding target(s) rather than on the HSP72 itself. Even though thermal stress can cause binding of both HSP72 and the sHSPs, such binding occurs over clearly different temperature ranges, suggesting that either the factors determining binding are different between the two families of HSPs or that the relevant alterations to their respective targets occur at different temperatures. Finally, although the sHSPs in skeletal muscle can undergo phosphorylation, it does not appear to strongly affect their binding to targets, and its significance and importance remains unclear.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

REFERENCES


