Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans

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1Norwegian School of Sport Sciences, Oslo, Norway; 2Department of Sports Science, Aarhus University, Aarhus; 3Department of Molecular Muscle Biology, Copenhagen Muscle Research Centre, Rigshospitalet, Copenhagen; 4Department of Medical Biochemistry and Genetics, University of Copenhagen, Copenhagen, Denmark; and 4Department of Physical Education and Health, Örebro University, Örebro, Sweden

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Paulsen G, Vissing K, Kalhovde JM, Ugelstad I, Bayer ML, Kadi F, Schjerling P, Hallén J, Raastad T. Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. Am J Physiol Regul Integr Comp Physiol 293: R844–R853, 2007. First published May 23, 2007; doi:10.1152/ajpregu.00677.2006.—In this study the stress protein response to unaccustomed maximal eccentric exercise in humans was investigated. Eleven healthy males performed 300 maximal eccentric actions with the quadriceps muscle. Biopsies from vastus lateralis were collected at 30 min and 4, 8, 24, 96, and 168 h after exercise. Cellular regulation and localization of heat shock protein (HSP) 27, αB-crystallin, and HSP70 were analyzed by immunohistochemistry, ELISA technique, and Western blotting. Additionally, mRNA levels of HSP27, αB-crystallin, and HSP70 were quantified by Northern blotting. After exercise (30 min), 81 ± 8% of the myofibers showed strong HSP27 staining (P < 0.01) that gradually decreased during the following week. αB-Crystallin mimicked the changes observed in HSP27. After exercise (30 min), the ELISA analysis showed a 49 ± 13% reduction of the HSP27 level in the cytosolic fraction (P < 0.01), whereas Western blotting revealed a 15-fold increase of the HSP27 level in the myofibrillar fraction (P < 0.01). The cytosolic HSP70 level increased to 203 ± 37% of the control level 24 h after exercise (P < 0.05). After 4 days, myofibrillar-bound HSP70 had increased ~10-fold (P < 0.01) and was accompanied by strong staining on cross sections. mRNA levels of HSP27, αB-crystallin, and HSP70 were all elevated the first day after exercise (P < 0.01); HSP70 mRNA showed the largest increase (20-fold at 8 h). HSP27 and αB-crystallin seemed to respond immediately to maximal eccentric exercise by binding to cytoskeletal/myofibrillar proteins, probably to function as stabilizers of disrupted myofibrillar structures. Later, mRNA and total HSP protein levels, especially HSP70, increased, indicating that HSPs play a role in skeletal muscle recovery and remodeling/adaptation processes to high-force exercise. To counteract harmful events and agents, skeletal muscle cells provide protective mechanisms. Stress proteins, such as heat shock protein 27 and 70 (HSP27 and HSP70, respectively) and αB-crystallin, seem to have a important function as stabilizers of the myofibrillar structure, since they appear to “catch” denatured proteins before they irreversibly aggregate and subsequently refold them to original form (4, 37, 45). Furthermore, skeletal muscle contains an arsenal of antioxidant systems that seem to be closely allied with the HSPs (22, 39). During stress, the activation of heat shock factor 1 (HSF1) will lead to increased levels of HSP mRNA and HSP molecules, rendering the cell more resistant to future harmful stressors (29).

The cytoskeletal/myofibrillar-protective effects of the small HSPs, i.e., HSP27 and αB-crystallin, are described in various cell types and models (35). Koh (22) hypothesized that the small HSPs function as cytoskeleton-protective proteins under eccentric exercise, in particular that HSP27 and αB-crystallin bind/translocate to Z-disk-related structures (such as actin and desmin) and prevent disruption of the cytoskeleton during high-force exercise. In support of this hypothesis, Koh and Escobedo (23) reported translocation of HSP25 (homolog to HSP27 in humans) and αB-crystallin to the Z-disk in rat muscle after eccentric exercise. Similarly, Golenhofen et al. (14) showed in a study on rats that HSP25 and αB-crystallin translocate and accumulate in the I-band after ischemic stress in skeletal muscle. To our knowledge, translocation of the small HSPs in human skeletal muscle after high-force exercise has not been reported. However, Fischer et al. (11) observed positive staining for HSP27 and αB-crystallin on cross sections in patients suffering from different myopathies. Increased mRNA and protein levels of HSP27, HSP70, and ubiquitin in human skeletal muscle after high-force eccentric exercise have been reported in recent studies (6, 7, 42, 47–50, 57), with the interpretation that the HSPs seem to be involved in remodeling and adaptation processes after high-force exercise (6, 48). In contrast to HSP27 and αB-crystallin, an acute HSP70 response is also seen after nondamaging, aerobic exercise protocols (20, 33).

The HSP response appears not to be restricted to a single bout of unaccustomed exercise, since Thompson et al. (47) observed a HSP27 and HSP70 response following a repeated bout of high-force eccentric exercise (4 wk after the first bout). Thompson et al. found, however, lower basal levels of these stress proteins; muscle damage; eccentric actions; skeletal muscle

MYOFIBRILLAR DISORGANIZATION and membrane disruption are hallmarks of exercise-induced muscle damage (24, 32), and the functional outcome of such muscle damage is reduced force-generating capacity (55). Subsequent inflammatory events in the exercised muscles, such as infiltration of blood-borne leukocytes, may be necessary for repair, regeneration, and adaptation, but the process itself may be transiently damaging because of release of reactive oxygen species from the infiltrated cells (51, 52).
HSPs in both control and exercised samples before the second bout. This somewhat contrasts the augmented levels of HSPs seen with normal training. Liu et al. (26–28) observed changes in HSP70 levels in response to rowing training and found an exercise intensity–dependent increase of HSP70. Gjøvaag and Dahl (12) observed increased protein levels of HSP27 and HSP70 in triceps brachii muscle after 5–8 wk of low- and high-intensity resistance training in untrained humans.

Previous human studies may have one or more limitations, such as: few included biopsy time points, HSP analyses conducted in only one fraction (e.g., the cytosolic fraction) of the homogenated muscle tissue, and no application of more qualitative methods, such as immunohistochemistry. This limits the possibility of observing rapid changes in the HSP response and movement of the HSP molecules between the cytosolic compartment and cytoskeletal/myofibrillar structures. Furthermore, in previous studies, the HSP response has not been investigated with respect to changes in muscle function, i.e., changes in maximal force-generating capacity.

In the present study, HSP27, αB-crystallin, and HSP70 responses are measured both qualitatively and quantitatively at multiple time points after high-force exercise. We hypothesized that a single bout of unaccustomed maximal eccentric exercise stimulates an acute HSP27 and αB-crystallin translocation to myofibrillar structures during exercise, with a subsequent increase in mRNA and protein levels for HSP27, αB-crystallin, and HSP70.

MATERIALS AND METHODS

Subjects. Eleven healthy male students (28 ± 4 yr, 180 ± 8 cm, 83 ± 6 kg; means ± SD) gave informed consent to participate in the study. The subjects' activity levels varied from sedentary to physically active, but none of the subjects was engaged in weight lifting or other forms of heavy strength training. The subjects were instructed not to take any form of medications or supplements (such as antioxidants). The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway.

Experimental design. A bout of maximal voluntary eccentric exercise was carried out with one leg; dominant or nondominant was randomly chosen. The other leg served as control in all tests before and after exercise. Biopsies were collected at 30 min and 4, 8, 24, 96, and 168 h after exercise (Fig. 1). The time points were chosen to get a picture of the early events (0.5–24 h) and the delayed response (96 and 168 h) to high-force eccentric exercise. To reduce the stress on subjects and minimize possible contamination from repeated biopsies, each subject provided biopsies at four of the six time points. This means that biopsies from a total of seven subjects could be analyzed at each time point. Recovery of muscle function was assessed with repeated tests of maximal concentric knee extensions. The first test was performed before the exercise bout to establish baseline values; the second test started ~3 min after the exercise bout, and further tests were carried out daily for 1 wk (Fig. 1). All subjects participated in four familiarization test sessions on separate days before participating in the study.

Exercise protocol. The subjects performed 300 unilateral, maximal, isokinetic, eccentric actions (30°/s) with the quadriceps muscle on a Cybex 6000 (Lumex, Ronkonkoma, NY). The subjects sat with 90° in the hip joints, fastened with seat belts, and arms held crossed over the chest. The range of motion was 35–105° (0° equals full extension of the knee joint). The workout lasted 40 min and consisted of 30 sets of 10 repetitions (30 s rest between each set).

Maximal force-generating capacity. Force-generating capacity was measured as maximal voluntary isokinetic concentric knee extension peak torque at 60°/s. The intraindividual coefficient of variation (CV) was <5%.

Biopsy procedure. A 5-mm Pelomi needle (Albertslund, Denmark) with manual suction was used to obtain tissue samples (3 × 30–100 mg) from the midsection of the vastus lateralis muscle. Subjects lay supine position while the procedure was performed under local anesthesia (Xylocain adrenaline, 10 mg/ml + 5 µg/ml; AstraZeneca). For the four biopsies, each needle insertion was placed 3 cm proximal to the previous insertion to avoid affected tissue from earlier biopsies. Muscle samples were rinsed in saline before visible fat and connective tissue were removed. Samples were divided and selected for immunohistochemistry, homogenization, or mRNA isolation. The samples for immunohistochemistry and homogenization were subsequently frozen in isopentane on dry ice and stored at −80°C until analysis. Samples for mRNA isolation were placed in RNA later (catalog no. 7024; Ambion) and stored at −20°C until further analysis.

Immunohistochemical staining on cross sections. Serial cross sections (5 µm) were cut with a cryostat microtome (Microm, Walldorf, Germany) at −22°C and mounted on Superfrost Plus microscope slides (Menzel-Gläser), air-dried, and stored at −80°C until further analysis. HSP27, αB-crystallin, and HSP70 were analyzed using respective antibodies (Table 1). To investigate colocalization, cross sections were double stained with antibodies against HSP27 and αB-crystallin and HSP27 and HSP70.

Muscle sections were rinsed 2 × 10 min in PBS and blocked for 30–60 min with normal 5% horse serum or 10% goat serum, together with 1% BSA and Triton X-100 (0.5%) in PBS. Sections were incubated with the primary antibody diluted in blocking solution for 2 h at room temperature or overnight at 4°C. Slides were washed in PBS 3 × 10 min and incubated for 60 min with an appropriate secondary antibody (fluorochrome-labeled or biotinylated; Table 1). The fluorochrome-stained sections were then washed 3 × 10 min in PBS and mounted with cover slips (Fluoromount-G; Chemi-Teknik). Diaminobenzidine-stained sections were developed (according to the manufacturer’s instruction; Table 1), dehydrated (2 min in 70, 90, and 100% ethanol), and coverslipped (Mountex; Histolab Production).

Images were captured using a digital camera (Axiocam HRC; Zeiss, Oberkochen, Germany) connected to an Axioskop-2 light microscope (Zeiss) with appropriate filters. Pictures were taken with ×20, ×40, and ×100 magnification.

The total number of counted fibers per section varied from 68 to 403 (mean value of 207 fibers). Only whole fibers were counted, i.e., damaged or partly damaged fibers in the core or especially around the edge of the samples were excluded. Fibers with overtly altered

![Fig. 1. Time line, with time points for tests and measurements.](http://ajpregu.physiology.org/lookup/doi/10.1152/ajpregu.00310.2007)
staining pattern (staining above background) were counted as “positive” fibers. Values are given as percentage positive fibers of the total number of counted fibers. There was normally no staining in the control samples, and identifying positive fibers in the exercise muscle was generally unproblematic (see Fig. 3).

ELISA and Western blotting. Muscle samples (50 mg) were homogenized in 5–10 vol of buffer containing a cocktail of protease inhibitors (P8340, Sigma) and centrifuged at 13,500 g for 15 min (15). The supernatant, representing the cytosolic fraction, was collected, and the remaining pellet was resuspended in phosphate buffer (0.02 M phosphate buffer, 140 mM NaCl, pH 7.4). After incubation (10 min at room temperature), samples were centrifuged at 3,000 g for 10 min at 4°C, resuspended in 0.02 M phosphate buffer, and then centrifuged at 3,000 g. This process was repeated twice. The final pellet was dissolved in 2× Laemmli sample buffer, and protein concentration was quantified by DC DC protein assay (catalog no. 500–0120; Bio-Rad; CV <10%). Protein concentration in the cytosolic fraction was measured with a BCA Protein Assay Kit (catalog no. 23227; CV <10%) after removing interfering substances with a Compat-Able Protein Assay Preparation Reagent Set (catalog no. 23215; both from Pierce). Samples were analyzed in duplicate and diluted 1:10 or 1:20 (CV <10%).

HSP27 in the cytosolic fraction was measured with a homemade double-sandwich ELISA using a monoclonal capture antibody, 25 ng protein/well (mouse anti-human HSP27; catalog no. SPA-800; Stressgen), and a polyclonal detection antibody diluted 1:5,000 (rabbit anti-human HSP27; catalog no. SPA-803; Stressgen). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Amxex, catalog no. RPN4301; Amersham) was used as a secondary antibody. Solid phase was high-binding polystyrene plates (catalog no. 3590; Costar), and tetramethylbenzidine (catalog no. S22128; Amersham) was used as substrate. Recombinant HSP27 (catalog no. SPP-715; Stressgen) was used as standards (1.56–25 ng/ml). All samples were analyzed in triplicate and diluted 1:100 or 1:300 (1–3 μg total protein), and optical density was read at 450 nm (CV <10%). The results from the homemade ELISA correlated highly (r = 0.92) with the commercial HSP27 kit from Stressgen (catalog no. EKS-500).

After homogenization, the HSP70 in the cytosolic fraction was measured with a commercial ELISA kit according to the manufacturer’s instructions (catalog no. EKS-700; Stressgen). All samples were analyzed in duplicate and diluted 1:50 or 1:100 (3–6 μg total protein; CV <10%).

Protein levels in the soluble (cytosolic) and insoluble (pellet) fractions were analyzed by Western blotting. Equal amounts of protein (20 μg/ml) were separated on 10% SDS-PAGE gels for 60 min at 200 volts and transferred to polyvinylidene difluoride membranes in TG buffer (Bio-Rad) for 60 min at 100 volts. After transfer, membranes were stained with Ponceau S to evaluate loading and transfer conditions and then blocked with 5% nonfat milk in 20 mM Tris, pH 7.5, 500 mM NaCl, and 0.1% Tween overnight. After being washed, the membranes were incubated for 2 h with a primary antibody against HSP27 and HSP70 (both 1:4,000, catalog no. SPA-800 and SPA-810, respectively; Stressgen). Blots were washed and then incubated with a secondary antibody conjugated to horseradish peroxidase (1:750; Pierce). After a final wash, protein bands were detected using chemiluminescence (SuperSignal West Dura; Pierce), and finally signal density was measured using a Kodak image station (Kodak 2000R, with Kodak 1D analysis software).

Note that the cytosolic protein levels of HSP27 and HSP70 were analyzed with the ELISA method at all six time points, whereas biopsy samples from 0.5 and 96 h after exercise were chosen for Western blotting. The main reason of choosing only two time points for Western blotting was because this method is only semiquantitative and not suitable for tracking small changes over time. Moreover, we were also restricted by the amount of tissue obtained from each subject at each time point.

The insoluble (pellet) fraction of homogenated muscle tissue contains the cell membrane, nuclear structures, the cytoskeleton and the contractile proteins, but, since myofibrillar proteins constitute >90% of the proteins in this fraction, we use henceforth the term “myofibrillar fraction.” The “cytosolic fraction” is used for describing the soluble fraction.

mRNA isolation and analysis. Total RNA was prepared based on a modified version of the single-step method described by Chomczynsk and Sacchi (2). Briefly, 10–20 mg of muscle tissue was homogenized in 1 ml of Trizol (Invitrogen) using a Polytron homogenizer (Kinematics, Luzern, Switzerland). After a short incubation at room temperature (5–10 min), 100 μl of 1–bromo-3-chloropropane was added to each sample, mixed, and incubated for 15 min at room temperature. Samples were centrifuged at 12,000 g for 15 min at 4°C, and the clear top phase was transferred to a new tube. Equal volumes of isopropanol were added, and the samples were left at room temperature for 10 min. To facilitate precipitation and visibility, 2 l of 1 M sodium acetate (pH 5.5) were added, and the samples were air-dried, and RNA was dissolved in 20 μl RNase-free water followed by 260/280 absorbance measurements. To verify quality of RNA, all samples were run on an Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay; Agilent Technologies). All samples scored an RNA integrity number of 7.8 or better.

mRNA levels were determined by Northern blotting using cloned PCR products as previously described (19). Briefly, specific primers were used to produce templates by PCR (see Table 2 for details), which were subsequently transcribed in the presence of [α-32P]dATP to produce radiolabeled probes (3,000 Ci/mmol). RNA samples were loaded at 200 ng/well on a denaturing formaldehyde agarose gel and run for 65 min at 7.5 V/cm. The gel was stained with SYBR Green II (Cambrex) and viewed on a fluorescence scanner for evaluation of RNA integrity. The RNA was then blotted to a nylon membrane (Positive; Appligene) and stored at −20°C until further use. Probes were diluted to a final concentration of 2 × 105 counts/(min·ml), and hybridization was performed overnight. Signals were captured on a phosphor-imager and analyzed by the producer’s software (Quantity One; Bio-Rad).

The time points selected for the mRNA analyses were 0.5, 4, 8, and 24 h after exercise. The 28S probe was made by 5′-phosphorylation of an oligonucleotide complementary to 28S rRNA (TCG CCG TTA CTG GAA TCC TGG TTA GTT TCT TT) using T4 polynucleotide kinase and [γ-32P]dATP. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was chosen as the internal control, assuming GAPDH mRNA to be constitutively expressed. To test this

### Table 1. Antibodies used in immunohistochemical staining on cross sections

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Dilution</th>
<th>Secondary Antibodies and Reactants</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP27 (monoclonal; Stressgen, catalog no. SPA-800)</td>
<td>1:100</td>
<td>Vector BA-2000 (horse anti-mouse); Vectastain ABC reagent and diaminobenzidine substrate kit for peroxidase (Vector SK-4100)</td>
<td>1:200</td>
</tr>
<tr>
<td>HSP27 (polyclonal; Stressgen, catalog no. SPA-803)</td>
<td>1:200</td>
<td>Alexa Fluor 568 (goat anti-rabbit)</td>
<td>1:200</td>
</tr>
<tr>
<td>HSP70 (monoclonal; Stressgen, catalog no. SPA-810)</td>
<td>1:200</td>
<td>Alexa Fluor 488 (goat anti-mouse)</td>
<td>1:200</td>
</tr>
<tr>
<td>αB-crystallin (monoclonal; Stressgen, catalog no. SPA-222)</td>
<td>1:5,000</td>
<td>Alexa Fluor 488 (goat anti-mouse)</td>
<td>1:200</td>
</tr>
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HSP, heat shock protein.
assumption, another unrelated “constitutive” RNA, 28S rRNA, was measured and normalized for GAPDH (43).

**Blood sampling and creatine kinase activity.** Blood was drawn from an antecubital vein in a 10-ml serum vacutainer tube before and repeated times after the exercise protocol (Fig. 1). After coagulating for 30–45 min at room temperature (20°C), the blood was centrifuged at 2,700 g for 10 min at 4°C. Serum was then immediately pipetted into Eppendorf tubes and stored at −80°C until analysis. Creatine kinase (CK) activity was analyzed with the Hitachi 917 Automated Biochemistry Analyzer (Roche, Basel, Switzerland), analytic CV being <2.8%.

**Muscle soreness.** Muscle soreness was rated on a visual analog scale (VAS) where 0 represented “not sore at all” and 100 mm “extremely sore” (Fig. 1). The subjects stretched, contracted, and palpated the quadriceps muscle to assess soreness in the vastus medialis and vastus lateralis muscles and centrally, including the rectus femoris and vastus intermedius muscles.

**Statistics.** A one-way repeated-measures ANOVA with Dunnett’s post hoc test was performed to identify significant changes in force-generating capacity. Because each of the 11 subjects provided muscle samples at four out of six time points (n = 7 on each time point), Student’s paired t-test or Wilcoxon signed rank test was used to analyze differences between the exercise and control muscle for all variables analyzed on muscle tissue. Selected bivariate relationships were examined with the Pearson product-moment correlation coefficient test or the Spearman rank correlation test. A P value of ≤0.05 was used for establishing statistical significance. mRNA data were log transformed before statistical analysis and presented as geometric means ± back-transformed SE. All other data are presented as means ± SE.

**RESULTS**

**Muscle function.** The maximal concentric torque at 60°/s was reduced by 47 ± 5% immediately after exercise (P < 0.01) and recovered slowly over the test week. After 23 h, the force-generating capacity was reduced by 35 ± 6%, and 1 wk after exercise the torque was still 13 ± 4% below the preexercise value (P < 0.01).

**Immunohistochemical analyses.** Half an hour after exercise, 81 ± 8% of the fibers from the exercised leg showed strong staining for HSP27 compared with only 0.9 ± 0.4% in the control leg (P < 0.01; Fig. 2). The portion of HSP27-stained fibers declined gradually thereafter, and after 96 h there was no significant difference between the exercised and control muscle (Figs. 2 and 3). However, large individual differences were observed: in one of the subjects (subject 8), for example, 68% of the counted fibers still showed strong HSP27 staining in the exercised muscle 96 h after exercise.

The proportion of HSP27 positive fibers correlated with the impairment of force-generating capacity, i.e., the individual area under the curve for the proportion of HSP27 positive cells correlated with the area under the curve for force-generating capacity (r = 0.80; P < 0.01).

The HSP27 staining was stronger in some fibers than others and tended to cluster, giving the fibers a granular appearance (Fig. 4). The staining indicates that HSP27 first and foremost binds to myofibrillar structures and the surrounding cytoskeleton. We observed no preferential staining to the cell membrane. However, in fibers with strong staining, the stained areas often included staining in the membrane.

The αB-crystallin staining mimicked the HSP27 response closely and colocalized with HSP27 in fibers with apparent myofibrillar disturbances (Fig. 5).

The number of HSP70 positive fibers in exercised muscle increased during the first 8 h to 23 ± 10% and stayed elevated at this level throughout the test week (P < 0.05; Fig. 2). At early time points, 0.5–24 h after exercise, the HSP70 staining was relatively weak compared with HSP27 and αB-crystallin, but resembled and colocalized with the small HSPs. At later time points (96 and 168 h), the staining became more evenly distributed and stronger (Fig. 3). At 96 h after exercise, fibers positive for both HSP27 and HSP70 were only observed clearly in one subject (subject 8): ~35% of the HSP70 positive fibers were also positive for HSP27. As described for HSP27, individual differences were also observed in the HSP70 staining. In three subjects, we observed only a few stained fibers at any time point (<6% of the counted fibers), whereas fibers from the other eight subjects showed staining at all time points. At late time points (96 and 168 h after exercise), when the staining was qualitatively strongest, the number of HSP70 positive fibers ranged from 25 to 49% of the counted fibers.
among the eight “responders.” The proportion of fibers positive for HSP70 correlated highly \( r = 0.94, P < 0.01 \) with reduced force-generating capacity 1 wk after exercise.

Weak or no staining was found in control muscle samples for any of the stress protein antibodies.

**HSP levels in cytosol.** We observed that the cytosolic (soluble) level of HSP27 in the exercised muscle, measured by the ELISA technique, was reduced to 51 ± 13% of the level in the control muscle 0.5 h after exercise \( (P < 0.01) \). Thereafter, the cytosolic HSP27 level progressively increased, and 96 h after exercise it tended to be higher in the exercised muscle compared with the control muscle \( (33 ± 17\%; P = 0.10) \); the individual peak HSP27 levels were significantly higher in the exercised muscle compared with the control.

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**Fig. 3.** Representative images of cross sections from vastus lateralis muscle stained for HSP27 and HSP70 from 6 time points. The HSP27 staining shows strongest immunoreactivity at the first time point and a rapid, gradual decrease in staining in the hours after exercise. The HSP70 staining shows positive fibers at all time points, but the staining is clearly strongest at 96 and 168 h after exercise. Note that the images are from 3 different subjects (2 from each); the exercised-control pairs are from the same subject. Scale bar = 100 μm.
muscle (61 ± 17%; P < 0.01). Western blotting for HSP27 in the exercised muscle showed 29 ± 9% higher levels in exercised muscle than the control at 96 h after exercise (P < 0.01).

The individual reductions in force-generating capacity immediately after exercise correlated with the reductions in the level of cytosolic HSP27 0.5 h after exercise (r = 0.86; P < 0.05; Fig. 7).

The HSP70 level in the cytosol tended to be lower in the exercised muscle 0.5 h after exercise (P = 0.09), but increased thereafter and was 203 ± 37% of the level in the control muscle 24 h after exercise (P < 0.05; Fig. 6).

The individual changes in force-generating capacity immediately after exercise correlated with peak values of HSP70 (r = 0.76; P < 0.01; Fig. 7).

**HSP levels in the myofibrillar fraction.** The HSP27 protein level in the myofibrillar (insoluble) fraction was ~15-fold higher in the exercised muscle than the control muscle 0.5 h after exercise (P < 0.01; Fig. 8). At 96 h after exercise, there was still approximately eight times more HSP27 bound to myofibrillar structures in the exercised muscle (P < 0.05).

Half an hour after exercise, myofibrillar-bound HSP70 was approximately twofold higher in the exercised muscle compared with control, and after 96 h this ratio was ~10 (P < 0.01; Fig. 8).

The main finding in this study was that maximal eccentric exercise caused a rapid translocation of small HSPs to myofibrillar proteins, indicated by the immunohistochemical staining pattern and HSP protein levels in the cytosolic and myofibrillar fraction. In addition, one bout of eccentric exercise increased the mRNA and later the protein levels of HSP70 in the days following exercise, and the HSP response seemed to be related to changes in muscle function.

A limitation with the presented study, which should be kept in mind when reading the following discussion, is that each subject delivered four biopsies, whereas biopsies were collected at six different time points, meaning that specimens...
from only seven out of eleven subjects could be analyzed at each time point.

Translocation and accumulation of small HSPs to myofibrillar structures. Immediately after exercise, we observed strong staining for HSP27 and αB-crystallin on cross sections, with a concomitant increase in myofibrillar-bound HSP27 and decrease in levels of cytosolic HSP27. These findings are in line with those of Koh and Escobedo (23) who studied the immediate HSP response in rat muscle after eccentric exercise. Like Koh and Escobedo (23), we believe that our observations indicate a translocation of cytosolic small HSPs to stressed, i.e., denatured, and damaged proteins in the cytoskeleton.

The positive correlation between reduced cytosolic HSP27 levels and reductions in force-generating capacity supports the assumption that HSP27 translocation is related to muscle damage. Reduced force-generating capacity is known to reflect muscle damage (55), and, accordingly, we observed a strong correlation between the degree of fibers showing ultrastructural changes (electron microscope analyses) and the degree of reduced force-generating capacity \( r = 0.91, P < 0.01; \) Raasstad T, Gylterud Owe S, Paulsen G, Enns D, Overgaard K, Cameri R, Kil S, Belcastro A, Bergersen L, Hallén J, unpublished observation). Thus the degree of myofibrillar disorganization seems to be associated with the degree of HSP27 translocation. This supports a statement by Fischer et al. (11) regarding myopathies that HSP27 and αB-crystallin are sensitive markers for focal loss of myofibrillar organization.

At early time points (0.5–24 h after exercise), staining on cross sections for HSP27, αB-crystallin, and HSP70 showed a variable degree of granular appearance. We believe that this granular staining pattern reflects the accumulation of HSPs to disrupted sarcomeres, which occurs throughout the length of the fiber. Preliminary data using confocal imaging of whole fibers supports this hypothesis, showing that HSP27 is found to accumulate specifically at disrupted sarcomeres, which become prominent when double stained with phalloidin. Furthermore, we observed colocalization of HSP27 and αB-crystallin, which agrees with the findings of Koh and Escobedo (23). Even if these small HSPs bind to areas of myofibrillar disorganization, it is not known to which protein(s) the stress proteins preferentially bind. All denatured proteins may be a target, but potential candidates are actin, M-line proteins, titin, and intermediate proteins, particularly desmin (11, 13, 35, 41). Further studies should be carried out to identify these proteins.

The strong immunohistochemical HSP27 and αB-crystallin staining at early time points could be interpreted as increased amounts of these small HSPs. However, we believe that this primarily reflects an accumulation of small HSPs in damaged/disrupted areas, not increased protein amounts. One possible explanation for the increased immunoreactivity could be due to reduced size of small HSPs oligomers, which potentially could increase the numbers of available epitopes for the antibody to bind. Reduced small HSP oligomer size is observed after eccentric exercise (23). It is unlikely that there is a detectable increased synthesis of small HSPs during exercise, keeping in mind that the exercise lasted for only 40 min and that the mRNA levels was not increased until 4 h after exercise. We have no available data on influx and efflux of HSP molecules, but movement of HSPs over the sarcolemma could potentially have affected our results. Increased CK activity in serum indicates altered membrane permeability or membrane damage; consequently, we could assume an efflux of HSPs.

Translocation of small HSPs seems to be related to damaged structures in myofibers, but do they only translocate to disrupted sarcomeres? Increased staining for phosphorylated HSP27 in biopsies from horses obtained within 10 min after apparently nondamaging aerobic exercise is reported by Von Ginneken et al. (53). However, Koh and Escobedo (23) found

Fig. 7. Left: correlation between changes in force-generating capacity and changes in the HSP27 level 0.5 h after exercise in the cytosolic fraction of homogenated muscle tissue from vastus lateralis muscle; \( n = 7 \). Right: correlation between changes in force-generating capacity and peak HSP70 levels in the cytosolic fraction of homogenated muscle tissue from vastus lateralis muscle; \( n = 11 \).
no HSP response to 75 nondamaging isometric contractions in rat muscles. This discrepancy is difficult to explain, but differences between species, exercise protocol, and use of different antibodies have to be considered. Nevertheless, HSP27 is known to both prevent critical denaturation and to “hold” already denatured proteins to aver pregnancy aggregation and render subsequent refolding or degradation by HSP70 and the ubiquitin-proteasome system, respectively (4, 5, 16). However, it is difficult to imagine how HSP molecules could bind to myofibrillar proteins if these proteins do not denature to some extent. It may be that, even during apparently nondamaging aerobic exercise, some denaturation of myofibrillar proteins occurs, and the binding of small HSPs has a prophylactic effect against further denaturation and protein damage. Consequently, the small HSPs may therefore detach soon, within minutes or a few hours, after exercise that cause only minor, reversible, protein denaturation/damage. Conversely, during eccentric, muscle-damaging exercise, more extensive denaturation and damage to cytoskeletal/myofibrillar proteins will cause stronger accumulation and more persistent binding of small HSPs. This is supported by our finding that the detachment of HSP27, indicated by reduced immunohistochemical staining, appeared to be faster in the subjects who showed fast recovery of muscle function and slower in subjects demonstrating slow recovery.

HSP70 immunoreactivity on cross sections. In contrast to Koh and Escobedo (23), we observed a rapid increase of HSP70 in the myofibrillar (insoluble) fraction of homogenated tissue. Furthermore, we report a granular immunohistochemical stain of HSP70 on cross sections. The HSP70 staining on samples from early biopsies (obtained 0.5–24 h after exercise), was, however, weak compared with that of HSP27 and αB-crystallin, but HSP70 and HSP27 seem to colocalize. No statistically significant reduction in the cytosolic fraction, a modest increase in the myofibrillar fraction, and general weak HSP70 staining indicate that HSP70 to a much lesser extent than the small HSPs translocate and accumulate to the cytoskeletal/myofibrillar proteins during muscle-damaging exercise. At later time points (96 and 168 h after exercise), there was no consistent colocalization with the small HSPs on cross sections, which could reflect a more general upregulation of HSP70 in fibers undergoing recovery and/or remodeling/adaptation. Possibly, there is an immediate fast and massive accumulation of HSP27 (and to some extent HSP70) to cytoskeletal/myofibrillar proteins in damaged fibers. Thereafter, the HSP27 molecules detach gradually, simultaneously with an increased generation of HSP70 molecules. This could explain why some fibers stain positive only for HSP27 at early time points, some are HSP27 and HSP70 positive (fibers in “transition phase”), and some only positive for HSP70 at late time points, when fibers are in the final phase of recovery.

Gene expression and increased protein levels of the small HSPs and HSP70. An increased amount of HSP27 and HSP70 on both the mRNA and protein level after eccentric exercise has been found by others (47, 48, 50). We found increased mRNA levels of HSP27 and αB-crystallin, but our data do not support a substantial increase in protein levels of HSP27. Because this has been clearly shown by Thompson et al. (47) and Feasson et al. (6), it is possible we missed the time point when the HSP72 levels peaked (we had no biopsies taken at 48 and 72 h). Conversely, the marked increased HSP70 mRNA levels did more convincingly precede the significant increase in the HSP70 protein level.

HSP70 mRNA. Noteworthy, several different genes for HSP70 exist (25), but our probe only recognizes HSP70A (HSP70-1) and HSP70C (HSP70-2), collectively referred to as HSP72. These isoforms have, however, repeatedly been reported to undergo the regulation in skeletal muscle in response to exercise (27, 54, 56). Thompson et al. (48) measured HSP70A, HSP70B, and HSP70C and found only increased HSP70C in biceps brachii muscle after maximal eccentric exercise, and only HSP70B to be increased in vastus lateralis muscle after downhill running. Regulation of the different HSP70 genes warrants further investigation, and we cannot exclude the possibility that other HSP70 isoforms than the ones we have probed for could be induced by our exercise intervention.

HSP70 protein levels. HSP70 seems to be involved in recovery and remodeling/adaptation processes in skeletal muscles (25, 31). In support of this assumption, we found increased protein levels of HSP70 in the days after exercise and a positive correlation between reduced force-generating capacity immediately after exercise and the increased HSP70 levels.
Thus, with increasing initial damage, correspondingly more HSP70 for refolding of denatured proteins and for correct folding of newly synthesized proteins is required (36, 46, 48). The increased amount of HSP70 molecules found in the myofibrillar fraction at 96 h after exercise could indicate that HSP70 play a role in the assembly and incorporation of new proteins in the myofibrillar framework. Alternatively, or additionally, the increased HSP70 levels could be important for protecting the muscle tissue from exercise-induced inflammation (31). The protection against exercise-induced inflammation can be expedient because the inflammatory process can be unnecessarily strong and actually cause increased (secondary) damage in the muscle tissue (30, 51). In particular, neutrophils release proteases and reactive oxygen species (ROS), and HSPs are, together with antioxidative systems, known to protect cells against oxidative stress (22, 39). Furthermore, HSP70 expression is stimulated by cytokines (8), and HSP27 and HSP70 seem able to protect cells against adverse effects of proinflammatory cytokines, such as tumor necrosis factor-α (17). In fact, we observed an increased accumulation of leukocytes, quantified and localized by radiolabeled leukocytes and immunohistochemical staining for CD16+ and CD68+ cells (Paulsen et al., unpublished observation). On the other hand, it has been shown that HSP70 has proinflammatory properties when released to the extracellular space (1), leaving the possibility that increased HSP70 expression in combination with membrane damage and/or release of HSP70 exosomes induced an increased immune response in the exercised muscle tissue. Thus the role of the HSPs in exercised-induced inflammation should be further elucidated.

It should be emphasized that the kind of muscle damage and inflammation that is associated with high-force eccentric exercise seems to be just one of several mechanisms that initiates an HSP70 response. HSP70 levels are, in contrast to the small HSPs, reported to increase markedly 2–7 days after aerobic exercise without any apparent signs of muscle damage (i.e., reduced force-generating capacity; see Refs. 20 and 33). This might indicate that metabolic perturbations (e.g., low pH), oxidative stress, and/or increased muscle temperature are of importance in enhancing HSP70 expression (9, 25). In contrast, one could expect that mechanical stress is the major stimulus during high-force eccentric exercise because of the fact that maximal eccentric exercise is less metabolically challenging than maximal concentric exercise, even though the force-generating capacity is greater during eccentric actions (44). Thompson et al. (48) observed increased expression of HSP27 and HSP70 concomitant with mitogen-activated protein kinase activation after high-force eccentric exercise, but not after downhill running, suggesting a mechanically sensitive pathway. Increased muscle temperature is also found to stimulate HSP70 expression in animal studies (38), although a recent study by Morton et al. (34) indicated that temperature per se is not of major importance during exercise in humans. Oxidative stress is a more plausible candidate since it has been observed that antioxidant administration can block the HSP70 response after aerobic exercise (10, 18, 21). Furthermore, the HSP transcription factor 1 (HSF1) is known to be redox sensitive (39). Oxidative stress during exercise could also be of importance in high-force eccentric exercise because mechanical distentions are important activators of plasma membrane systems for generation of superoxide and nitric oxide (39). However, the majority of ROS generation after eccentric exercise seems delayed by one or more days after exercise and is possibly related to an inflammatory process (3, 39). Because of the observation of a positive correlation between changes in force-generating capacity and HSP70 protein increase, and knowing that the subjects with large reduction in maximal force-generating capacity were the same subjects who had the strongest inflammatory response, our data indicate that large mechanical stress, muscle damage, and inflammation are the main stimuli for augmented HSP expression in our exercise protocol.

**Conclusion.** HSP27 and αB-crystallin rapidly and transiently bind and accumulate on the myofibrils during unaccustomed maximal eccentric exercise, presumably to protect the myofibrillar filament organization. The clustering of HSP27 and αB-crystallin in regions of the fibers indicates that some myofibrils or segments of myofibrils are damaged or critically stressed, whereas other myofibrils are intact. In the hours and days after exercise, the synthesis of HSP70 is increased, probably to serve in the recovery and remodeling/adaptation processes that protect the muscles fibers during subsequent high-force exercise.

**REFERENCES**


