TITLE

Subcellular movement and expression of HSP27, αB-crystallin, and HSP70 after two bouts eccentric exercise in humans

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RUNNING TITLE: Heat shock protein response to eccentric exercise

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The aims of this study were to investigate the sarcomeric accumulation and expression of heat shock proteins (HSPs) after two bouts of maximal eccentric exercise. Twenty four subjects performed two bouts of 70 maximal voluntary eccentric actions using the elbow flexors in one arm. The bouts were separated by three weeks. The changes in concentric (60°·s⁻¹) and isometric (90°) force-generating capacity were monitored for nine days after each bout, and biopsies were taken one and 48 hours and four and seven days after bout 1 and one and 48 hours after bout 2. The content of HSP27, αB-crystallin, HSP70, and desmin in the cytosolic and cytoskeleton/myofibrilar fraction of homogenized muscle samples was determined by immunoassays, and the cellular and subcellular localization of the HSPs in the myofibrilar structure was analyzed by conventional and confocal immunofluorescence microscopy and quantitative electron microscopy. The force-generating capacity was reduced by ~50% and did not recover completely during the three weeks following bout 1. After bout 2, the subjects recovered within one week. The HSP levels increased in the cytosolic fraction after bout 1, especially HSP70 (~300% 2-7 days after exercise). Increased levels of HSP27, αB-crystallin, and HSP70 were found in the cytoskeletal/myofibrilar fraction after both bouts – despite reduced damage after bout 2. At the ultrastructural level, HSP27 and αB-crystallin accumulated in Z-disks, in intermediate desmin-like structures (αB-crystallin), and in areas of myofibrilar disruption. In conclusion, HSP27 and αB-crystallin accumulated in myofibrilar structures, especially in the Z-disks and the intermediate structures (desmin). The function of the small HSPs is possibly to stabilize and protect the myofibrilar structures during and after unaccustomed eccentric exercise. The large amount of HSP27, αB-crystallin, and HSP70 in the cytoskeletal/myofibrilar fraction after a repeated bout of exercise suggests a protective role as part of the repeated-bout effect.
INTRODUCTION

Heat shock proteins (HSPs), and especially HSP70, have been found to be up-regulated in exercised muscles in the days after both non-damaging aerobic exercise (19; 31) and muscle-damaging, high-force eccentric exercise (9; 42; 43). The physiological rationale for augmented HSP levels in myofibers in response to intensive exercise is probably to increase protection against future situations of oxidative and mechanical stress (18; 20; 27; 34). In high-force eccentric exercise, the cytoprotective role of HSP27 and αB-crystallin has been indicated by translocation of these small HSPs from the cytosolic compartment to cytoskeletal/myofibrilar structures during exercise (21; 38). Allegedly they stabilize and prevent damage to these structures during the mechanical strain of high-force eccentric exercise (20). Hitherto, this exercise-induced translocation of the small HSPs has not been verified at the ultrastructural level by use of electron microscopy, and it is not known to which sarcomeric structures the HSPs bind, although the Z-disks have been suggested (21).

While a bout of unaccustomed eccentric exercise can cause myofibrilar disruptions and severe, long-lasting muscle weakness, the repeated-bout is considerably better tolerated (32; 33). This has been named the “repeated-bout effect” (7). Although several studies have attempted to elucidate the mechanisms behind the repeated-bout effect, this feature of skeletal muscle adaptation is still not fully understood (30). High-force eccentric exercise initiates disruptions in the myofibrilar ultrastructure that correlate with the reduced force-generating capacity in the days following exercise (8; 14; 39). If a cause-effect relationship exists, a remodeling and reinforcement of the myofibrilar structures would render it more resilient in the face of future bouts of exercise. In fact, several studies have found increased content of certain structural proteins, such as desmin, and indications of increased numbers of sarcomeres in series after eccentric exercise and training (2; 9; 26; 49). In line with this, it seems intuitively appropriate to reinforce the protection systems of HSPs as well. Up-regulated levels of the HSPs would imply more HSP molecules to stabilize the cytoskeletal/myofibrilar structures during exercise and to
facilitate recovery and remodeling by refolding of damaged proteins and folding *de novo* synthesized proteins after exercise (15; 16; 20). Indeed, it has been demonstrated that transgenic over-expression of HSP70 gave increased protection against muscle damage from eccentric (lengthening) actions and improved recovery in old mice (29). Thus, it seems plausible that HSPs are involved in the repeated-bout effect (30). Until now, the role of HSPs in the repeated-bout effect has only been investigated in two human studies (41; 46).

Thompson *et al.* (41) investigated the response of HSP27 and HSP70 in stressed muscles (elbow flexors) after two bouts of eccentric exercise, separated by four weeks. The repeated-bout effect was successfully initiated and manifested by attenuated exercised-induced muscle weakness and soreness, but no change in the HSP response was found. The total cellular content of both HSP27 and HSP70 was increased in relatively similar magnitude 48 hours after the first and second bouts. Surprisingly, however, the basal levels of HSP27 and HSP70 appeared to be lower before the second bout. This finding casts doubt on the HSPs as important players in the repeated-bout effect. Vissing *et al.* (46) found a blunted translocation of HSP27 to cytoskeletal structures three hours after a repeated bout of eccentric step exercise, performed eight weeks after the first bout. However, no changes in the cytosolic levels of HSP27 or HSP70 were observed after the first bout. The lack of HSP up-regulation could possibly be due to relatively low degree of muscle damage inflicted during the exercise protocol. Accordingly, no indications for a role of the HSP system in the repeated-bout effect were found.

The primary aim of this study was to investigate the translocation from the cytosolic compartment to the myofibrillar/cytoskeletal structures and the expression of HSP27, αB-crystallin, and HSP70 after an unaccustomed bout of maximal eccentric exercise and after a repeated bout. With respect to previous studies (41; 46), we applied a highly muscle-damaging exercise protocol to increase the chances of an up-regulation of the HSP protein levels. Because the repeated-bout effect subsides with time (5; 35), we restricted the period between...
bouts to three weeks. Moreover, we applied both qualitative and quantitative immunoassays on subcellular fractions of the exercised muscles in order to elucidate more sides of the expected HSP response. The secondary aim was to study the binding of the small HSPs to sarcomeric structures at the ultrastructural level by quantitative electron microscopy (immunogold).

We hypothesized that 1) an unaccustomed bout of maximal eccentric exercise would cause muscle damage and increase the cellular levels of HSP27, αB-crystallin, and HSP70, 2) the increased amount of these HSPs would be associated with the repeated-bout effect, and 3) the small HSPs (HSP27 and αB-crystallin) would be associated with Z-disks and disrupted areas at the ultrastructural level.
METHODS

Subjects

Twenty-four healthy subjects (17 men and 7 women; 21-37 years of age) gave written, informed consent to participate in the study. The subjects were physically active and involved in different activities, such as running, cycling, and team sports. None of the subjects were familiar with maximal eccentric exercise with the elbow flexors. No exercise was performed for three days prior to the experiment. The subjects were asked to continue their regular diet and to not take any form of medication or prescription-free supplements (such as anti-oxidants). They were also instructed to avoid exercise, stretching and massage therapy (etc.) for the duration of the investigation. The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway.

Study design

The subjects performed two bouts of eccentric exercise (bout 1 and bout 2), separated by three weeks, using either the dominant or the non-dominant arm (randomly chosen). The same arm was exercised at both bouts, and the other arm served as a non-exercised control for all tests and measurements.

Prior to the exercise bouts and for nine days following each bout, muscle function (force-generating capacity) was measured as voluntary maximal concentric and isometric torque, and a blood sample was drawn. Biopsies from m. biceps brachii were collected from both the exercised arm and control arm at one and 48 hours, as well as four and seven days (96 and 168 hours) after bout 1, and one and 48 hours after bout 2. Each subject was scheduled to have biopsies taken at three out of the six biopsy time points. Two subjects declined to undergo the biopsy procedure after the first biopsy and one subject declined after the second biopsy, due to the physical discomfort of the procedure. One subject was biopsied only once due to technical circumstances.
The subjects participating in this experiment were part of an encompassing study investigating the effect of a cyclooxygenase-2 (COX-2) inhibitor (celecoxib; Paulsen et al. (37)). Ten subjects were administered the COX-2 inhibitor for nine days after bout 1, while the other 14 subjects served as a placebo group. No drug was administered after bout 2. No differences in change in force-generating capacity (the main variable) were found (37), and there were no detectable differences in the HSP response (presented below). Consequently, the combined results from both groups are presented herein.

Both females and males were recruited to this study. Taking into account that only seven of the 24 subjects were females and that we found no clear gender differences, we only present the combined data from both genders.

Samples from five of the subjects (three females and two males) that demonstrated considerably reduced force-generating capacity (greater than the group average) and clearly increased HSP staining on cross-sections (see below) were chosen for ultrastructural analyses (immunogold).

**Exercise protocol**

For the eccentric exercise, the subjects were positioned in a chair and fastened with belts over the hip, chest, and shoulder. The upper arm was supported by a cushion (Technogym, REV 9000, Gambettola, Italy). Thus, the shoulder joint was kept in a slightly flexed position (30-35° from the vertical axis) and prevented from moving during the elbow exercise. The subjects gripped a handle connected to the lever arm of the dynamometer. Since the handle could be rotated about the longitudinal axis, the subjects were instructed to supinate their forearm (elbow joint) for maximal activation of m. biceps brachii. The exercise protocol consisted of 14 x 5 repetitions of eccentric actions with the elbow flexors, with 30-35 seconds rest between
sets. The lever arm of the dynamometer automatically returns to the starting point, so no
muscle force was necessary for the elbow flexion phase. The range of motion (ROM) in the
elbow joint was 40-175° (180° = full extension) and the velocity was 30°·sec^{-1}. The subjects
were verbally encouraged to resist maximally through the whole ROM and they received real
time visual feedback on their performance on a computer screen.

The peak torque (Nm) and the work (J) of each eccentric action were registered and total work
for the exercise was calculated. All values registered during exercise and tests (see below)
were corrected for gravity and passive tension.

**Muscle function, changes in force-generating capacity**

In the same position as during the exercise, maximal force-generating capacity was measured
as peak torque during three consecutive maximal, isokinetic, concentric elbow flexions at 60°·s^{-1}
(ROM: 175-40°) and peak torque during isometric actions at 90° in the elbow joint (five-
second actions; two attempts; Technogym, REV 9000). All subjects completed 1-2
familiarization tests on separate days before they entered the study, and two pre-tests were
preformed on day one of the experiment. The mean of the two pre-test values was used as
baseline value. Subjects always warmed up their elbow muscles by arm cranking (three min at
30-50 W) before tests, and four submaximal concentric actions were included in the test
protocol of the dynamometer (Technogym, REV 9000). The intra-individual coefficient of
variation (CV) for the force-generating capacity measurements was < 5%.

**Biopsies**

A 5 or 6 mm Pelomi-needle (Albertslund, Denmark) with manual suction was used to obtain
tissue samples (usually 2-3 x 50-100 mg) from the midsection of m. biceps brachii. During the
biopsy, subjects lay supine, and the procedure was performed under local anesthesia
(Xylocain® adrenaline, 10 mg·ml^{-1} + 5 μg·ml^{-1}; AstraZeneca, Södertälje, Sweden). The first
needle incision was placed near the midline of m. biceps brachii, and the second and third
incisions were placed approximately 1-2 cm medially and laterally to the first incision. Care was
taken to avoid affected tissue from earlier biopsies. The muscle samples were rinsed in
physiological saline and visible fat and connective tissue were removed. Samples for cross-
section immunohistochemistry and homogenization (for protein measurements) were frozen in
isopentane on dry ice and stored at -80°C until analysis. Samples for single-fiber
immunocytochemistry were placed in RNALater (no. 7024, Ambion LTD Huntingdon,
Cambridgeshire, UK) and stored at -20°C (not frozen), while samples for electron microscopy
immunocytochemistry were placed in a fixation solution (4% paraformaldehyde and 0.1%
glutaraldehyde in 0.1 M phosphate-buffered saline [PBS]) and stored at 4°C.

With the exception of four subjects (mentioned above), each subject had three biopsies taken
at three of the six biopsy time points, from both the exercised arm and the control arm: One
hour (n = 9), 48 hours (n = 16), four days (n = 10), and seven days (n = 10) after bout 1, and
one hour (n = 9) and 48 hours (n = 11) after bout 2. We obtained only three biopsies from the
same muscle to limit the risk of contamination from previous biopsies.

Protein levels

Western blotting of HSP27, αB-crystallin and HSP70

Muscle samples (50 mg) were homogenized using a subcellular extraction kit according to the
supplier’s instructions (ProteoExtract Subcellular Proteome Extraction Kit, Merckbiosciences,
no. 539790, Darmstadt, Germany). Cytosolic, membrane, nuclear, and cytoskeletal fractions
were obtained after stepwise extraction: i.e., the homogenate was incubated in four different
buffers followed by centrifugation. Protein content was determined using the RC/CD Protein
Assay kit I (Bio-Rad, no. 00-0121, San Diego, CA, USA). The purity of the cytosolic and
cytoskeletal fractions was tested with antibodies against GAPDH (glyceraldehyde-3-phosphate
dehydrogenase) and desmin (Table 1). Desmin was detected in the cytoskeletal (myofibrillar)
fraction, but not in the cytosolic fraction, whereas GAPDH was found in the cytosolic fraction, but not in the cytoskeletal fraction.

Equal amounts of protein (10-20 μg per well) were separated on NuPage 4-12% Bis-Tris Mini Gels (NP 0321 box, Invitrogen, Carlsbad, CA, USA) for ~35 min at 200 V and transferred to PVDF membranes (using NuPage reagents and buffers). After transfer, membranes were stained with Ponceau S and gels were stained with Coomassie Blue (all unspecified reagents were from Bio-Rad Laboratories Inc., Hercules, CA, USA). Membranes were then blocked with 5% skimmed milk powder in TBST (Tris-buffered saline and 0.1% Tween20) overnight. After washing, the membranes were incubated for two hours with primary antibodies in 1% skimmed milk powder in TBST (Table 1). Blots were washed and then incubated with a horseradish peroxidase conjugated secondary antibody (Table 1). After a final wash, protein bands were visualized using chemiluminescence (SuperSignal West Dura, no. 34076, Pierce Biotechnology, Rockford, IL, USA), and signal density was measured using a Kodak image station (Kodak 2000R, with Kodak 1D analysis software, Rochester, NY, USA).

Note that the HSP27, αB-crystallin and HSP70 cytosolic protein levels are expressed in percentage (%) of the cytosolic control values, and the levels in the cytoskeletal fraction are expressed in percentage (%) of cytosolic control values and normalized for cytoskeletal control values. The reason for this was that in some runs we could not detect bands for the HSPs in the control cytoskeletal fraction.

**ELISA of HSP27**

HSP27 in the cytosolic and cytoskeletal fraction were measured with a homemade double antibody sandwich ELISA, utilizing a monoclonal capture antibody (25 ng per well), a polyclonal detection antibody, and a horseradish peroxidase conjugated secondary antibody (Table 1). The assay was performed in high-binding polystyrene microtiter plates (no. 3590, Costar, Corning, NY, USA). Tetramethylbenzidine (no. CL07 Calbiochem, Merck KGaA,
Darmstadt, Germany) was used as substrate and 2 N Sulphuric acid was used as stop solution. Recombinant HSP27 (no. SPP-715, Stressgen Bioreagents, Ann Arbor, MI, USA) was used as standards (0.78-25 ng·ml⁻¹). All samples were analyzed in triplicate and diluted 1:100 or 1:300 (~1-3 µg total protein), and O.D. was read at 450 nm (analytic CV < 10%).

**Light microscopic immunofluorescence on cross-sections**

Serial muscle cross-sections (7 µm) were incubated with the primary antibodies (as specified in Table 1), and diluted in blocking solution (10% newborn calf serum, 1% bovine serum albumin [BSA] and 0.05% Tween20 in TBS [TBST]) for two hours at room temperature or overnight at 4°C. The sections were incubated for 45-60 min with an appropriate secondary antibody (fluorochrome-labeled; Table 1) and mounted with coverslips (Fluoromount-G, Chemi-Teknik AS, Oslo, Norway, or ProLong Gold Antifade Reagent with DAPI, Invitrogen-Molecular Probes, Eugene, OR, USA). The muscle sections were washed in TBST between each step.

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

The mean number of counted fibers per section was ~200 (range 35-540). All whole fibers on each sample were counted. Damaged or partly damaged fibers, located at the edge of the samples, were excluded. Fibers with overtly altered staining pattern (staining above background) were counted as “positive” fibers. Values for positive fibers are given as percentage of the total number of counted fibers.
Light and confocal microscopy immunofluorescence on whole fiber preparations

Single fibers were teased under dissecting microscope and in RNAlater, before being blocked and permeabilized overnight (50 mM glycine, 0.04% saponin, 0.25% BSA in 0.01M PBS). Fibers were incubated with gentle agitation overnight at room temperature with primary antibodies (Table 1). Fibers were subsequently incubated with appropriate secondary antibodies for two hours at room temperature (Table 1). The fibers were washed 3 x 1 hour in 1% BSA in PBST (0.5% Triton-X-100) between antibody incubations and before mounting with coverslips.

Stained whole fiber segments were examined through both a standard fluorescent microscope (as described above) and a confocal microscope (TCS-SP, Leica, Mannheim, Germany; equipped with an Ar\textsuperscript{/}Kr\textsuperscript{+} ion laser).

This examination was carried out to get a qualitative impression of the staining pattern of the small HSPs over larger areas of the exercise muscle fibers (fiber length ~2-6 mm). Fibers from subjects (n = 5) that demonstrated strong staining on cross-sections one and 48 hours after exercise were selected for this analysis. Fibers from the non-exercised control muscle were always included in the analysis.

Note that phalloidin, which preferentially binds to F-actin, normally stains in a regular striated pattern, but when sarcomeres are disrupted, the phalloidin staining appears more intense. Thus, damaged areas become visible.
Quantitative electron microscopy; immunogold analysis

The tissue samples (fixed with 4% paraformaldehyde and 0.1% glutaraldehyde) were embedded in Lowicryl HM20 resin using a freeze substitution technique as previously described (3). Embedded tissue samples were longitudinally oriented before ultrathin sections (80 nm) were cut on a Reichert-Jung ultramicrotome (Reichert Ultracut S, Vienna, Austria) using a diamond knife (Ultra 45°, Diatome Ltd., Biel, Switzerland), and mounted on coated nickel grids (500 mesh grids and using a Coat-Quick “G” adhesive pen [Electron Microscopic Sciences, Fort Washington, USA]).

Immunogold labeling was performed as described in Bergersen et al. (3), and using primary antibodies against HSP27, αB-crystallin and desmin (Table 1). In brief, ultrathin sections were etched in 2% hydrogen peroxide in 0.1 M phosphate buffer (PB) to remove resin (20 min), followed by a rinse in PB. Free aldehyde groups in the fixed tissue were neutralized by incubating sections 10 min in sodium borohydride (0.1%) and glycine (50 mM) dissolved in TBST (0.005 M Tris, 0.9% NaCl, 0.1% Triton X-100, pH 7.4), followed by rinsing in TBST. Sections were incubated in a blocking solution for 10 min (2% BSA, 2% human serum and 0.2% skimmed milk powder in TBST) before incubation with primary antibodies diluted in TBST with 2% BSA overnight at room temperature. The following day sections were rinsed in TBST and incubated with the appropriate secondary antibodies coupled to either 10 or 15 nm gold particles for 60 min (Table 1). Finally, sections were rinsed in distilled H₂O, air dried, and counterstained in uranylacetate (5% solution in 40% ethanol) and lead citrate (0.3%). For analysis, a FEI Tecnai 12 transmission electron microscope (Hillsboro, OR, USA) equipped with a digital camera was used.

The amount of abnormalities in the myofibrillar structure (at each time point) was quantified as described in Lauritzen et al. (22). Herein we define a fiber with myofibrillar disruptions as a fiber with three or more adjacent disorganized sarcomeres (in series of parallel).
On immunogold stained sections from the exercised muscle, disrupted regions were first localized at low primary magnification (1700x-2550x). Magnification was then increased (26500x) to detect gold particles representing the different proteins, and one picture containing damaged sarcomeres, as well as one picture of an adjacent intact area, were taken. The procedure was repeated for 10 fibers from each sample. In control muscles where the morphology was primarily intact, only intact areas were investigated.

In intact areas in exercised and control muscles, myofibers were divided into A-bands, I-bands, Z-disks, mitochondria and cytosolic compartments. Surface area was measured using analySIS Pro (Soft Imaging System, Münster, Germany) and the number of gold particles was counted in each compartment. In disrupted regions it was not possible to separate different sarcomeric compartments, so a disrupted area was treated as one compartment. Labeling density is given as gold particles per square micrometer (gp·[µm²]⁻¹).

**Blood sampling and myoglobin analysis**

Blood was drawn from an antecubital vein into a 10 ml serum vacutainer tube. After coagulating for 30-45 min at room temperature, the blood was centrifuged at 2700 g for 10 min at 4°C. Serum was immediately pipetted into Eppendorf tubes and stored at -80°C until analysis. Myoglobin was analyzed with an automated chemistry analyzer (Modular E, Hitachi High- Technologies Corporation, Tokyo, Japan); analytic CV being < 6%.
Statistics

To identify statistically significant changes in force-generating capacity from baseline, a one-way repeated measures ANOVA with Dunnett’s post-hoc test were performed. We assessed the differences from baseline for both exercised and control arms, as well as differences between the arms. Changes in myoglobin were evaluated by Friedman’s test with Dunn’s post-hoc test. For changes in the force-generating capacity and serum myoglobin (log-transformed), a two-way ANOVA (with Bonferroni’s post hoc test) was used to assess differences between bouts for the exercised arm. Because different subjects were biopsied at each time point, a paired Student’s t-test was used to evaluate differences detected in biochemical and histological analyses (exercised vs. control). Data sets encompassing large individual variations and skewed distribution were log-transformed before being evaluated with a paired Student’s t-test. An unpaired Student’s t-test or a Mann-Whitney test was applied for testing differences between subgroups (i.e., celecoxib vs. placebo and males vs. females). Selected bivariate relationships were examined with the Pearson product-moment correlation coefficient test or Spearman rank correlation test. P ≤ 0.05 was used for establishing statistical significance. Data are presented as means and standard error of the mean (SEM), if not otherwise stated in the text. SEM was chosen (in preference to standard deviation) to increase the readability of the figures and because comparing the mean values was an important aspect of the data analysis. The statistics were performed using Microsoft® Excel 2003 (including statistiXL 1.8), InStat® 3.06, Prism® 5.01 and Statemate™ 2.0 (GraphPad Software Inc., San Diego, CA, USA).
RESULTS

Exercise protocols and muscle function

The total work performed in bout 1 and bout 2 was -4750 ± 320 and -4720 ± 300 J, respectively. Eccentric peak torque (during the exercise protocol) was reduced by 45 ± 2% and 33 ± 2% in bout 1 and bout 2, respectively (p < 0.01 between bouts). Thus, the initial peak torque (in set 1) was somewhat lower in bout 2, but total work was the same for both bouts since the decrease in eccentric force-generating capacity was smaller during bout 2 than bout 1.

Maximal concentric (60°·s⁻¹) and isometric (90°) torques were decreased by 45 ± 2% and 51 ± 2% immediately after bout 1, respectively (p < 0.01; Figure 1). During bout 2, the maximal concentric and isometric torques decreased by 34 ± 2% and 41± 2%, respectively (p < 0.01; Figure 1). The force-generating capacity was not fully recovered three weeks after bout 1, but normalized one week after bout 2. The acute loss of concentric and isometric force-generating capacity was smaller after bout 2 than bout 1, and recovery occurred within four days (96 hours; Figure 1). The force-generating capacity of the control arm did not change during the experiment.

Myoglobin in serum

The myoglobin concentration in serum was elevated one and eight hours and three, four, and seven days after bout 1, and one and eight hours after bout 2 (p ≤ 0.05; Figure 1). The individual reductions in isometric force-generating capacity correlated with serum myoglobin levels (r = 0.81; three days [72 hours] after bout 1; n = 24; p < 0.01).
Protein levels in homogenized muscle

Western blotting of the small HSPs

One hour after bout 1, the HSP27 and αB-crystallin levels were uniformly reduced in the cytosolic fraction and concomitantly increased in the cytoskeletal fraction of exercised muscles, compared to control muscles (p < 0.01; Figure 2). Thereafter, the cytosolic levels increased and were elevated compared to control in the days following bout 1, while the protein content in the cytoskeletal fraction stayed elevated (especially αB-crystallin). One hour after bout 2, the cytoskeletal levels of the small HSPs were increased (p < 0.01), but the cytosolic levels were not significantly reduced. Note that the HSP27 and αB-crystallin levels in the cytoskeletal fraction one hour after bout 1 and bout 2 were comparable, whereas the decrease in the cytosolic fraction of HSP27 and αB-crystallin was less evident after bout 2 than bout 1 (p = 0.05 for both).

Western blotting of HSP70

The levels of HSP70 in the cytosolic and cytoskeletal fractions were higher in exercised muscles than in control muscles at all time points, except one hour after bout 1 (p ≤ 0.04; Figure 2). The cytosolic HSP70 levels were higher one hour after bout 2 than bout 1 (p = 0.03), and the levels in the cytoskeleton were higher both one and 48 hours after bout 2 than bout 1 (p ≤ 0.03).
Fractions of membrane and nuclei structures (preliminary results)

Western blot analyses of HSP27 and HSP70 (n = 6-14) in membrane and nuclei fractions showed increased protein levels for both HSPs at two, four and seven days after bout 1 and one and 48 hours after bout 2 (p = 0.3 - < 0.01). These findings indicate that the increased content found in the cytosolic fraction (2-7 days after exercise) was not due to movement of HSPs from other compartments of the myofibers. On the contrary, increased HSP levels in all fractions, i.e., the cytosolic, cytoskeletal, membrane and nuclei fraction, support a general up-regulation of the HSPs in the exercised myofibers.

Western blotting of desmin

The desmin levels in the cytoskeletal fraction of the exercised muscles and the control muscles were identical one and 48 hours and four days (96 hours) after bout 1. Seven days (168 hours) after bout 1, however, the desmin levels were increased by 20 ± 7% in the cytoskeletal fraction of the exercised muscles (p = 0.02). After bout 2, the desmin levels tended to be elevated one hour after exercise (22 ± 11%; p = 0.08), and were significantly higher than control 48 hours after exercise (14 ± 6%; p = 0.05).

HSP27 protein content determined by ELISA

The changes in HSP27 in the cytosolic and cytoskeletal fractions found by western blotting were verified by the ELISA method (Figure 3). Note, however, that the ELISA demonstrated a more gradual decrease in the cytoskeletal fraction (from one hour after exercise) than the western blot analysis. Comparable levels of HSP27 were found in the cytoskeletal fraction of the exercised muscles obtained one hour after bout 1 (679 ± 157 ng·mg⁻¹) and bout 2 (788 ± 244 ng·mg⁻¹). The values found in the cytosolic and cytoskeletal fractions in samples from the control muscle did not change over time: mean (± standard deviation [SD]) values for all control measurements were 2184 ± 687 ng·mg⁻¹ and 129 ± 167 ng·mg⁻¹ for the cytosolic and cytoskeletal fractions, respectively. The individual reductions of HSP27 in the cytosolic fraction one hour after bout 1 correlated with the reductions in the force-generating capacity.
immediately after exercise (r = 0.80; n = 9; p = 0.01). This correlation was not observed after bout 2.

<Figure 3>

**Light microscopic immunofluorescence on cross-sections**

**Small HSPs**

The number of HSP27 positive fibers observed was highest one hour after bout 1, and declined gradually thereafter (Figure 4). The same time course trend was found after bout 2, but the number HSP27 positive fibers was lower than in bout 1 (p = 0.18 at one hour and p < 0.01 at 48 hours). The staining pattern of the HSP27-antibody was non-uniform, scattered and granular (Figure 4). Qualitatively speaking, the areas of staining in each positive fiber were frequently fewer and smaller after bout 2 than bout 1. After bout 1 (but not after bout 2), the number of positive fibers one hour after exercise correlated with the reductions in force-generating capacity immediately after exercise (r = 0.79, n = 9; p = 0.02). Thus, the subjects with the largest reduction in force-generating capacity had the highest numbers of HSP27 positive fibers.

The αB-crystallin staining co-localized with HSP27 (Figure 4). However, the staining seemed to persist longer for αB-crystallin than for HSP27 (Figure 4 – see also Figure 2), especially in the subjects with the most pronounced muscle weakness. Normally, no scattered/granular staining pattern of the small HSPs was found on samples from the control muscle (Figure 4).
The number of fibers with high immunoreactivity (positive) for HSP70 increased from one to 48 hours, and stayed elevated four and seven days (96 and 168 hours) after bout 1 (compared to control; \( p \leq 0.05 \); Figure 4). After bout 2 the number of HSP70 positive fibers increased from one to 48 hours \( (p = 0.02) \), but the number of positive fibers was lower 48 hours after bout 2 than bout 1 \( (p = 0.03) \). One and 48 hours after exercise, the staining pattern of the HSP70-antibody tended to be scattered and granular, similar to the small HSPs (co-localization; Figure 4). However, four and seven days after exercise the HSP70 staining displayed a more intense and even cytoplasmic stain (Figure 4). No overt HSP70 staining was found on samples from the control muscle (Figure 4).

There was a relationship between the individual number of HSP70 positive fibers at one and 48 hours after bout 1 and the delta value of positive fibers between bouts (i.e., bout 1 minus bout 2; \( r = 0.86; n = 22; p < 0.01 \)). Hence, the subjects with many positive HSP70 fibers after bout 1 had considerably fewer positive fibers after bout 2, whereas those with few stained fibers after bout 1 had about the same or even more positive fibers after bout 2. The same tendency was seen for the small HSPs.

**Fiber type differences**

Of all fibers analyzed, 60 ± 10% (SD) were type II fibers (IIa + IIx; SC71 positive). One and 48 hours after exercise both type I and II fibers were positive for the small HSPs. Fibers positive for HSP70 four and seven days (96 and 168 hours) after exercise appeared to be primarily type II fibers. On samples from the control muscles, the HSP27 antibody often gave a slightly stronger cytoplasmic stain in type II than type I fibers, whereas the αB-crystallin antibody often gave a slightly more intense staining in type I fibers (Figure 4).

Necrotic myofibers (fibers that lacked dystrophin staining) were observed in about 1/3 of the subjects four and seven days after bout 1. The necrotic fibers were primarily type II fibers and
demonstrated increased staining intensity for HSP27 and αB-crystallin, but not for HSP70 (Figure 4). Small, apparently regenerated, fibers, with central nuclei were seen after bout 2 (three weeks after bout 1). Regenerated fibers were presumably a consequence of the necrosis seen after bout 1, and these fibers often displayed a relatively intense, cytoplasmic stain for HSP27 – but occasionally a scattered, granular stain as well. The regenerated fibers were primarily type II fibers.

**Light and confocal microscopy immunofluorescence on whole fiber preparations**

HSP27, αB-crystallin, and phalloidin all gave positive, though irregular, staining patterns on whole fiber preparations from exercised muscles (one and 48 hours after exercise). Large variations were observed both between fibers and along each individual segment of muscle fiber (Figure 5). The irregular staining patterns observed by conventional light microscopy were confirmed using confocal microscopy imaging. This clearly demonstrated defined areas of staining by both small HSPs, which closely co-localized with increased and irregular phalloidin staining (Figure 5C-D) and desmin (not shown). An interesting observation, pertaining to the shape of disruptions, was that damaged areas were typically wider than they were long, i.e., disruptions spread more laterally than longitudinally. Moreover, the staining pattern seen on whole fiber preparations (scanning through the fiber) supported the scattered, granular staining pattern seen on cross-sections.

Fibers from the control muscles demonstrated very weak staining of HSP27 and αB-crystallin (Figure 5B). With confocal microscopy, staining for both HSP27 and αB-crystallin were confined to the Z-disks area.

<Figure 5>
Quantitative electron microscopy

Muscle fibers obtained from the exercised muscle (from five subjects) demonstrated more myofibrilar disruptions than fibers from the control muscle (Figure 6). The proportion of fibers with myofibrilar disruptions (obtained one and 48 hours after exercise) was less after bout 2 than bout 1 (57 ± 10% vs. 94 ± 4% of the analyzed fibers, respectively; p = 0.02). A detailed description of the ultrastructural changes is presented in Lauritzen et al. (22).

Both HSP27 and αB-crystallin accumulated heavily in areas of myofibrilar disruptions (Figure 7). The small HSPs also accumulated in the Z-disks of apparently intact sarcomeres in the exercised muscles. No such accumulation was seen in control muscles. A higher density of gold particles in the Z-disks was observed together with decreased density of gold particles in the intermyofibrilar space for HSP27 (Figure 7G). A similar trend was seen for αB-crystallin, though it was less evident. However, there appeared to be, in total, more gold particles representing αB-crystallin in the samples from exercised muscles than in control muscles samples per unit of area (p = 0.01; Figure 7H). Moreover, some of the αB-crystallin staining in the intermyofibrilar space could be due to increased interaction between αB-crystallin and desmin. Additionally, in the control samples, the observed density of αB-crystallin was higher in the intermyofibrilar space than in the other sarcomeric areas/structures (p < 0.01). This was not the case in samples from the exercised muscles. Thus, compared to control, a higher proportion of αB-crystallin was found in the sarcomeric structures in the exercised muscles, particularly in the Z-disks. αB-crystallin accumulated to a greater extent than HSP27 in streamed Z-disks and Z-disks on the border between intact and disrupted areas. Streamed Z-disks and Z-disks next to disrupted sarcomeres frequently contained > 5 times more gold particles than Z-disks in control muscle (Figure 7I). In addition, αB-crystallin appeared to bind desmin in particular, as gold particles bound to the αB-crystallin-antibody were seen as “pearls
on a string” on structures that resembled desmin filaments (Figure 7C and E). Immunogold labeling of desmin supported this finding (Figure 7F).

The density of gold particles representing HSP27 in the intermyofibrilar space correlated with the reduction in isometric force-generating capacity one or 48 hours after bout 1 ($r = 0.91; n = 5; p = 0.03$), meaning that the subjects with the largest reduction in force-generating capacity had the largest reduction of HSP27 in the intermyofibrilar space, indicating translocation to the myofibrilar/cytoskeletal structures. Note that there was also a close relationship between the number of fibers with myofibrilar disruptions and changes in force-generating capacity ($r = 0.9; n = 5; p < 0.05$; Lauritzen et al. (22)).

Taken together, the immunogold staining pattern adds to the findings on cross-sections and whole fiber preparations analyzed with immunofluorescence. Moreover, the correlation between the ELISA HSP27 data and changes in force-generating capacity were supported by the relationships found between changes in force-generating capacity and the immunofluorescence and immunogold staining pattern of HSP27.

### Celecoxib (COX-2 inhibitor) vs. placebo

No differences in the HSP response were detected between the celecoxib group and the placebo group. As revealed by western blotting, the mean of individual peak increases in the cytosolic fraction (relative to control) were: $43 \pm 15\%$ vs. $49 \pm 16\%$ (HSP27), $102 \pm 24\%$ vs. $59 \pm 24\%$ ($\alpha$B-crystallin) and $188 \pm 33\%$ vs. $232 \pm 66\%$ (HSP70) for the celecoxib group and placebo group, respectively.
DISCUSSION

We observed a HSP response to an unaccustomed bout of maximal eccentric exercise and to a repeated bout. Although the HSP response was generally smaller after the second bout – in line with changes in muscle function, the high levels of HSP27, αB-crystallin, and HSP70 in the cytoskeletal/myofibrillar structures after the repeated bout suggest that the HSPs could play a role in the repeated-bout effect. We also present for the first time localization of the small HSPs at the ultrastructural level after eccentric exercise in humans. The small HSPs translocated and accumulated in areas of myofibrilar disruption and on sarcomeric structures – preferentially the Z-disks. A strength of this study was the consistent coherence of findings between the different immunological techniques used to investigate protein movement between cellular compartments and changes in protein content.

The HSP response to eccentric exercise

The HSP response to high-force eccentric exercise observed in the present study verifies previous findings (11; 21; 38; 42; 43; 46). The increased immunostaining for the small HSPs on cross-sections and segments of myofibers, especially one hour after exercise, indicates intracellular translocation and accumulation of the HSP molecules in and close to areas of myofibrilar disruptions, as confirmed at the ultrastructural level by immunogold staining. The increased levels of the small HSPs in the cytoskeletal fraction and the concomitant decrease in the cytosolic fraction one hour after exercise indicate a translocation of HSP molecules within the myofibers, and not, predominately, an influx of HSPs over the membrane or increased protein synthesis. Because we have previously observed increased mRNA levels of HSP27, αB-crystallin, and HSP70 three to 24 hours after eccentric exercise protocols (38; 46), it is likely that the increased protein content observed in the days after exercise was due to increased protein expression of these HSPs in the stressed myofibers.
On muscle cross-sections, the characteristic scattered, granular stain of the small HSPs (11; 38) was observed in both type I and II fibers, especially at early time points (one and 48 hours after exercise). In a study by Folkesson et al. (11), a scattered, granular staining of HSP27 was only seen in type II fibers after resistance exercise (70% of 1 RM; 10 x 8 repetitions), which indicate preferential stress (myofibrilar disruptions) on type II fibers. As our maximal eccentric protocol put the working muscles under considerably more mechanical stress than the resistance exercise protocol used by Folkesson et al., it seems reasonable that the type I fibers were affected as well in the present study. The present results support our previous findings of HSP27 staining in both type I and II fibers in samples from m. vastus lateralis after maximal eccentric exercise (38).

HSP70-positive fibers were observed in the exercised muscle at all time points, but the staining appeared particularly intense at two, four, and seven days after exercise. The HSP70-positive fibers observed 2-7 days after exercise were primarily type II fibers. This may indicate that the increased HSP70 staining reflects recovery, remodeling and adaptations, since type II fibers are more vulnerable to exercise-induced muscle damage than type I fibers (12; 17). The increased staining in the days after exercise signify increased levels of protein (38; 44), as supported by increased immunoblot levels of HSP70 in both the cytosolic and cytoskeletal fractions. In contrast to our observations, Tupling et al. (44) primarily found increased staining intensity of HSP70 in type I fibers. This discrepancy could be explained by differences in the exercise protocol – our maximal eccentric exercise vs. Tupling et al.’s isometric exercise (5-second action intervals at 60% of maximal isometric force for 30 minutes). Thus, there seems to be a differential HSP70 response, reflecting the different degree of stress put upon the type I and II fiber types in the two exercise protocols.
The repeated-bout effect

The repeated-bout effect observed was manifested by attenuated loss of force-generating capacity and faster recovery of muscle function after the second bout of exercise. Moreover, the serum myoglobin response was considerably attenuated after bout 2, compared to bout 1. A reduced amount of myofibrillar disruptions confirmed that less damage was inflicted during the repeated bout. Since we have previously observed an association between the reduction of force-generating capacity and translocation of HSP27 to cytoskeletal/myofibrilar structures (38), we expected the accumulation of HSP27, αB-crystallin, and HSP70 in the cytoskeletal/myofibrilar fraction to be lower after bout 2 than bout 1. However, the HSP levels in the cytoskeletal/myofibrilar fraction were similar or even higher after bout 2 than bout 1 (HSP70). This could indicate that these HSPs are involved in the increased protection and resilience of the cytoskeletal/myofibrilar structures during high-force exercise. A more efficient translocation of these HSPs is therefore plausibly a mechanism behind the repeated-bout effect.

Our data suggest increased total levels of the HSPs in the week following the first bout. Furthermore, because the reduction of the HSPs in the cytosolic fraction was generally smaller and the accumulation of HSPs in the cytoskeletal fraction tended to be larger one hour after bout 2 than bout 1, the total levels of HSPs were likely to have been higher during bout 2 than bout 1. Assumingly, higher levels of HSP could have provided greater protection against the stress applied in the second bout. In most subjects, we observed fewer fibers with intense, scattered, granular staining of the small HSPs on cross-sections after bout 2 than bout 1. Possibly, this reflected a decreased number and size of areas of disrupted sarcomeres and less distinct HSP accumulation. Taken together, this could mean that more of the HSPs in the cytoskeleton after bout 2 functioned to protect and stabilize rather than to rescue disrupted sarcomeres and damaged proteins – as was likely the case after bout 1. In other words, the
translocation and accumulation of the HSPs to the cytoskeleton was possibly more evenly
distributed in the myofibrilar/cytoskeletal structure after bout 2 than after bout 1.

Contrary to our findings, results from a study by Vissing et al. (46) did not point to a role for the
HSPs in the repeated-bout effect, as they observed a blunted translocation of HSP27 and
HSP70, which coincided with an attenuated loss in force-generating capacity after the second
bout. These differences could be explained by differences in the exercise protocols and the
time span between bouts (8 weeks vs. 3 weeks in the present study). Judged from the more
"mild" (~15%) reduction of the force-generating capacity after the first bout, their exercise
protocol was less damaging than that in the present study. Furthermore, Vissing et al. found no
evidence for increased levels of HSP27 and HSP70 (seven days after the first bout). Since the
repeated-bout effect decreases gradually with increasing time interval between bouts (5; 35), it
may be that the increased protective effect of the HSPs is markedly attenuated or gone after
eight weeks, especially if the initial damage is limited. Thompson et al. (41) reported a similar
relative increase of HSP27 and HSP70 two days after a second bout (four weeks after the first
bout), but, intriguingly, the protein levels in both the exercised muscle and control were lower
after the second bout than the first bout. This indicates reduced basal levels of HSPs before
the second bout, a finding that contradicts our observations. Comparable to our findings,
however, Thompson et al. (43) observed attenuated reduction of muscle function after the
second bout. Hence, this was not reflected in the relative HSP response, implying that the
extent of muscle damage was not the only determining factor behind this response. Based on
our results, the HSP response seems more regulated by the extent of muscle damage after the
first than the second bout of maximal eccentric exercise. Future studies should investigate
regulating mechanisms of the HPS response after exercise further.

In addition to increased accumulation of HSPs in the cytoskeletal fraction, we also observed an
increase in desmin content in the cytoskeleton one week after bout 1, and after bout 2. An
increase in desmin levels could indicate strengthening of the cytoskeletal and myofibrilar
structures, because desmin is an important intermediate filament at the Z-disks (6), which are susceptible to damage during and after eccentric exercise (1; 13). Other studies have found concurring results: Barash et al. (2) found increased desmin levels in rats 3-7 days after eccentric muscle work, and Feasson et al. (9) reported increased desmin levels together with HSP27 and αB-crystallin in humans 14 days after downhill running. Yu et al. (49; 50) suggested desmin as a central player in the remodeling process (sarcomerogenesis) after eccentric exercise. Moreover, rather than being a one-time response to unaccustomed exercise, the up-regulation of cytoskeletal proteins, such as desmin, appears to be a continuing adaptation to repeated bouts of high-force exercise (i.e., training; (24; 47; 48)). It is plausible that the strengthening of cytoskeletal/myofibrilar structures requires a concomitant up-regulation of certain HSPs, especially αB-crystallin, which is a part of these structures (6).

Taken together, our observations suggest that increased levels of HSP27, αB-crystallin, and HSP70 increase the protection of the cytoskeletal/myofibrilar structures during maximal eccentric exercise. In addition, our data point towards structural changes, including increased levels of the intermyofibrilar protein desmin that may render the muscles more robust and resilient in the face of mechanical forces. Collectively, these changes could be important elements in the repeated-bout effect after unaccustomed, high-force, eccentric exercise. However, the relative impact of these mechanisms is not known.

**Localization of the small HSPs in the myofibrilar structure**

There is little, albeit convincing, evidence for increased binding of the small HSPs to cytoskeletal/myofibrilar structures after high-force exercise (11; 21; 38; 46). However, this has not previously been verified at the ultrastructural level. Our results provide new insight as to where the small HSPs translocate via immunogold staining and electron microscopy. Although several cytoskeletal/myofibrilar proteins may be subject to damage and denaturation during and after high-force eccentric exercise, proteins of the Z-disk and the intermediate filament
proteins, e.g. desmin, have been suggested to be particularly vulnerable (13; 21; 25). Correspondingly, Z-disks and intermediate (desmin) filaments were the main structures in which the small HSPs accumulated. In areas of myofibrilar disruptions, in which the normal ultrastructure could not be easily identified, both HSP27 and αB-crystallin accumulated in particularly high numbers. Although, we were unable to elucidate the exact sarcomeric proteins/filaments to which the small HSPs bound in disrupted areas, the close relationship between HSP27 and actin suggests an interaction between these proteins (10; 23). αB-crystallin appeared to accumulate in areas of overt disruption/damage and in Z-disks next to disrupted sarcomeres more distinctively than HSP27. This might indicate that αB-crystallin is important in "sealing off" damaged areas. Moreover, αB-crystallin was also strongly associated with desmin-like structures (apparently in contrast to HSP27). A close relationship between αB-crystallin and the intermediate filament desmin was not surprising, since their interactions are well known from myopathies (10; 45). Immunoprecipitation (pulldown assays) of the small HSPs could be a promising procedure in future investigations of the protein interaction between the small HSPs and cytoskeletal/myofibrilar structures after exercise.

No effect of a COX-2 inhibitor

Half of the subjects in this study were administrated a COX-2 inhibitor (celecoxib), but no effects on the HSP response were found. Animal studies have provided evidence for adverse effects of the COX-2-inhibitors on muscle growth and recovery from severe muscle injuries (4; 36; 40), but we could not verify this in our human model (37). Our observations indicate that the COX-2 pathway is not of major importance for the HSP response after eccentric exercise. It should, however, be emphasised that with the number of subjects recruited and the large individual differences, more subtle differences may have stayed undetected.
Methodological considerations

A shortcoming in the present study is the lack of biopsies obtained just prior to bout 2, which would have given important information about the amount of HSPs “available” during bout 2 (which we presumed to be increased). Moreover, when obtaining multiple biopsies from the same muscle, there is a chance of obtaining tissue affected by previous biopsies (28). This “repeated-biopsy effect” should, however, be largely accounted for by comparing observations from the exercised muscle with those from the non-exercised, control muscle. To minimize the risk of sampling tissue affected by previous biopsies, each subject was biopsied at only three of the six time points. However, this reduced the number of samples to be analyzed on each time point and weakened the statistical power.
CONCLUSION

Two bouts of eccentric exercise resulted in a HSP response characterized by an immediate movement of the small HSP (HSP27 and αB-crystallin) from the cytosol to cytoskeletal/myofibrillar structures during exercise. The reduction in the cytosolic fraction (translocation) was less evident after the second than first bout. Despite less damage inflicted during the second than first bout, the levels of HSP27, αB-crystallin, and HSP70 in the cytoskeletal fraction were comparable or higher (HSP70) after the second bout of eccentric exercise. Indications of increased total amount of HSPs together with the high levels of HSPs in the cytoskeletal/myofibrillar structures led us to suggest that the HSPs provided increased protection against exercise-induced damage during and after the second bout. In addition, increased levels of desmin indicated strengthening of the myofibrilar structure per se. From immunogold staining and electron microscopy, we confirmed a translocation and accumulation of the small HSPs on cytoskeletal/myofibrillar structures. The small HSPs seemed to accumulate in the Z-disks and areas of disrupted sarcomeres. αB-crystallin was also closely associated with the intermediate filament desmin.
ACKNOWLEDGEMENT

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REFERENCE LIST


Table 1. Antibodies, dilutions, and incubation times according to the immunological assays used. IHC: Immunohistochemistry, ICC: immunocytochemistry.

Figure 1. Changes in force-generating capacity, measured as (A) voluntary maximal concentric torque (60°·s⁻¹) and (B) isometric torque (90°) after bout 1 and bout 2. C) Changes in the serum concentration of myoglobin after bout 1 and bout 2. N = 24; values are mean ± SEM. # denotes differences between exercised and baseline values, p ≤ 0.05. * denotes differences between bout 1 and bout 2, p ≤ 0.05.

Figure 2. Content of: A) HSP27, B) αB-crystallin, and C) HSP70 in the cytosolic and cytoskeletal fractions after bout 1 and bout 2, measured by western blotting. Note that the cytosolic levels are expressed as percentage (%) of control cytosolic levels and that the cytoskeletal levels are expressed relative to control cytosolic levels and normalized for the control cytoskeletal levels. N = 9-16 (at each time point); values are mean ± SEM. ¤ (cytosol) and # (cytoskeleton) denote differences between exercised and control samples, p ≤ 0.05. * denotes differences between bout 1 and bout 2, p ≤ 0.05.

Figure 3. Changes in the concentration of HSP27 in the cytosolic and cytoskeletal fractions after bout 1 and bout 2 as percentage (%) of control; measured by a homemade ELISA. N = 9-13 (at each time point); values are mean ± SEM. ¤ (cytosol) and # (cytoskeleton) denote differences between exercised and control samples, p ≤ 0.05. Note that for the cytosolic fraction, values from 48, 96 and 168 hours (two, four, and seven days) after exercise are combined to increase the statistical power in this analysis. For subjects with two values within this time period, the individual mean value was used.
Figure 4. Adjacent cross-sections (in rows) displaying immunoreactivity for αB-crystallin (green), HSP27 (green), and HSP70 (red), as well as SC71 (myosin heavy chain II; green) and dystrophin (red) plus DAPI (blue, nuclear stain). Note that HSP27 and HSP70 were stained on the same section. A, B, C marks the same fibers on all four images from the same time point (three serial cross-sections). Samples are from two group-representative subjects, encompassing six biopsy time points after bout 1 (one and 48 hours and four and seven days) and bout 2 (one and 48 hours). Samples from the non-exercised control muscle from one subject are shown in the bottom row. Note the strong, scattered, granular stain for HSP27 and αB-crystallin at one hour after exercise. HSP70 demonstrated here the most intense staining at seven days after exercise. The arrow on the seven days images points to a necrotic fiber (dystrophin negative). After bout 2 the staining for the HSPs was generally weaker. Scale bar = 100 μm. Graphs: Number of positive fibers for HSP27, αB-crystallin, and HSP70 (% of total number of counted myofibers) after bout 1 and bout 2. N = 9-15 at each time point; values are mean + SEM. # denotes differences between exercised muscle and control muscle values, p ≤ 0.05. * denotes differences between bout 1 and bout 2, p ≤ 0.05.

Figure 5A and B) Five (#1-5) segments of whole, single fibers from exercised muscle (obtained one hour after bout 1) demonstrating immunoreactivity for αB-crystallin (light micrographs). Note that the staining intensity varied considerably along the length of the myofibers and between fibers. Fiber segments from control muscle (#6 and #7) showed no such staining pattern. Scale bar = 100 μm. Figure 5C-E) Confocal micrographs of a segment of a whole fiber from exercised muscle (obtained one hour after bout 1) stained with phalloidin (C; green) and αB-crystallin (D; red); E) overlay. Arrows point to areas with disruption in the myofibrilar structure. Control fibers very infrequently demonstrated disruptions in the myofibrilar structure (not shown). Scale bar = 10 μm.
Figure 6. Electron micrographs of ultrastructural changes (disrupted sarcomeres) observed in a myofiber from an exercised muscle (left; obtained one hour after bout 1) and the intact structure of a control fiber (right). Scale bar = 2 μm.

Figure 7. Samples from exercised muscles (obtained one and 48 hours after bout 1) stained for HSP27, αB-crystallin, and desmin. Images A, C, E, and F are electron micrographs (EM), and B and D are confocal micrographs (CM). Note that the EM and CM images are not of the same myofibers. Arrows on the EM images (I-IV) point to gold particles labeling HSP27 and αB-crystallin. The arrows on image IV point to a Z-disk on the border of a disrupted sarcomere and to an intermediate filament (presumably desmin; see image E and F). Arrows on the CM images (B and D) show the directions of the fibers and areas of high immunoreactivity for the HSP27 and αB-crystallin antibodies. * marks Z-disks; Mito: mitochondria. Scale bars = 1 μm.

Images E and F show immunogold labeling of αB-crystallin and desmin. Arrows point to gold particles marking αB-crystallin and desmin; scale bar = 0.5 μm. Graphs: Quantification of gold particles labeling HSP27 (G) and αB-crystallin (H) in different areas of the myofibrilar structure. I) Frequency distribution of the number of gold particles labeling αB-crystallin in the Z-disks of fibers from exercised muscles and control muscles. The bars in G and H show mean values and full range. Mito: Mitochondria; IMS: Intermyofibrilar space; DS: Disrupted sarcomeres.
Table 1

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<tr>
<th>Primary antibodies</th>
<th>IHC (cross-sections) / ICC (whole fibers); light / confocal microscopy</th>
<th>ELISA</th>
<th>Western blotting</th>
<th>Immunogold; electron microscopy</th>
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<td>HSP27 (monoclonal; SPA-800, Stressgen Bioreagents, Ann Arbor, MI, USA)</td>
<td>IHC: 1:300; 2 hr at room temp.</td>
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<td>αB-crystallin (monoclonal; SPA-222, Stressgen Bioreagents)</td>
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<td>Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA, USA)</td>
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<td>Desmin (monoclonal; M0724, DAKO, Glostrup, Denmark)</td>
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<td>Desmin (polyclonal [goat], sc-7559, Santa Cruz Biotechnology, CA, USA)</td>
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<td>GAPDH (monoclonal, Ab9484, Abcam)</td>
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<td>SC71 (S. Schiaffino, University of Padova, Italy)</td>
<td>IHC: 1:500; overnight at 4°C</td>
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<tr>
<th>Secondary antibodies</th>
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<th>Dilution and incubation times</th>
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<td>Goat anti-mouse (Alexa Fluor® 488, Invitrogen)</td>
<td>IHC/ICC on cross-sections and whole fibers</td>
<td>IHC: 1:500; 45 min; ICC: 1:500; 2 hr; both at room temp.</td>
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<td>Goat anti-rabbit (Alexa Fluor® 488, Invitrogen)</td>
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<td>Goat anti-rabbit (Alexa Fluor® 568, Invitrogen)</td>
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<td>Goat anti-rabbit, horseradish peroxidase conjugated (RPN4301, Amdex, GE healthcare Life Sciences, Buckinghamshire, UK)</td>
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<td>Goat anti-mouse, horseradish peroxidase conjugated (no. 31430, Thermo Scientific, Rockford, IL, USA)</td>
<td>Western blotting</td>
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<td>Goat anti-rabbit 10 nm (no. 10735; BB International, Cardiff, UK)</td>
<td>ICC; immunogold</td>
<td>1:20; 1 hr at room temp.</td>
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<td>Donkey anti-goat 15 nm (DAG-51012/2, Aurion, Wageningen, The Netherlands)</td>
<td>ICC; immunogold</td>
<td>1:500 (HSP27) / 1000 (αB-crystallin); 1 hr at room temp.</td>
</tr>
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Figure 2

A  
HSP27 in cytosol (% of control cytosol)

Time (hours after exercise)

B  
αB-crystallin in cytosol (% of control cytosol)

Time (hours after exercise)

C  
HSP70 in cytosol (% of control cytosol)

Time (hours after exercise)
Figure 3
**Figure 4**

Bout 1

- **1 h**
- **2 d**
- **4 d**
- **7 d**

Bout 2

- **1 h**
- **2 d**

Control

**HSP27 positive myofibers (%)**

- Exercised
- Control

**HSP70 positive myofibers (%)**

- Exercised
- Control

**HSP70 positive myofibers (%)**

- Exercised
- Control

**αB-crystallin**

- Exercised
- Control

**αB-crystallin**

- Exercised
- Control

**αB-crystallin**

- Exercised
- Control
Figure 6

Exercised  Control
Figure 7