Heat shock protein translocation and expression response is attenuated in response to repeated eccentric exercise.

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Short title

HSP and repeated eccentric exercise.

ABSTRACT

Aim: This study hypothesised that heat shock protein (HSP) translocation and upregulation is more probable to occur after eccentric exercise than after concentric exercise or repeated eccentric exercise**.**

Methods: Fourteen young, healthy, untrained male subjects completed two bench-stepping exercise bouts with 8 weeks between bouts, and were compared to a control-group (n=6). Muscle biopsies collected from m. vastus lateralis of both legs prior to and at 3 hours, 24 hours and 7 days after exercise, were quantified for mRNA levels and/or for HSP27, αβ-crystallin and inducible HSP70 content in cytosolic and cytoskeletal protein fractions.

Results: The first bout of exercise reduced muscle strength and increased muscle soreness predominantly in the eccentric leg ($p<0.05$). These responses were attenuated after the repeated eccentric exercise bout ($p<0.05$), suggesting a repeated bout adaptation. Increases in inducible HSP70 and HSP27 protein content in cytoskeletal fractions were observed exclusively after eccentric exercise ($p<0.05$). For HSP27, a ~ 10-fold upregulation after first bout eccentric exercise was attenuated to a ~ 4-fold upregulation after the repeated eccentric exercise bout. mRNA levels for HSP70, HSP27 and αβ-crystallin were upregulated within ~ 2-9 fold ranges at time points 3 and 24 hours post exercise ($p<0.05$). This upregulation was induced exclusively by eccentric exercise but with a tendency to attenuated expression 3 hours after the repeated eccentric exercise bout.

Conclusion: Our results show that HSP translocation and expression responses are induced by muscle damaging exercise, and suggest that such HSP responses are closely related to the extent of muscle damage.

Key words: gene and protein expression, protein degradation, repeated bout effect, transcriptional regulation, protein translocation

INTRODUCTION

Muscles subjected to unaccustomed exercise, and especially eccentric exercise, are extremely prone to muscle damage (Clarkson & Hubal 2002, Paulsen et al. 2007). This muscle damage can be observed by electron microscopy, as sarcomeric disruptions, and also as a loss of myocellular membrane integrity attested by an increased efflux of muscle enzymes into the plasma and/or an increased influx of plasma-Ca^{2+} to the muscle cells (Belcastro et al. 1998, Friden et al. 1983, Morgan & Proske 2004, Proske & Morgan 2001). Damaged ultrastructural components are subjected to protein degradation by Ca^{2+}dependent and non-Ca^{2+}dependent proteolytic systems as well as attraction of immune-reactants, thus further contributing to temporary reductions in muscle strength and delayed onset of muscle soreness (DOMS) (Allen 2001, Jackman & Kandarian 2004, Nedergaard et al. 2007, Paulsen et al. 2005, Stupka et al. 2001, Vissing et al. 2008). Muscles subjected to a comparable unaccustomed concentric exercise modality do not exhibit a similar response (Vissing et al. 2008).

Repeated eccentric exercise is observed to attenuate, often quite significantly, the impairment in muscle function, enzyme leakage and muscle soreness development observed after the first exposure of the muscles to similar eccentric exercise. It has been observed that this adaptive response remains even if there are several months between the eccentric exercise bouts, and this response is commonly referred to as the repeated bout effect (McHugh 2003, Nosaka et al. 2001). Neural, mechanical and cellular

mechanisms are all speculated to be causally related to the development of a repeated bout effect (McHugh 2003). With regard to myocellular mechanisms, we have previously investigated the potential role of protein degradative systems. More specifically, we have investigated the hypothesis of attenuated Ca^{2+}calpain-calpastatin and/or ubiquitin-proteasome proteolytic system responses (Nedergaard et al. 2007, Vissing et al. 2008). Both proteolytic systems revealed several molecular candidates that responded exclusively to eccentric exercise, but there was no clear evidence that these candidates were strong determinants for the development of a repeated bout effect (Nedergaard et al. 2007, Vissing et al. 2008).

Another cellular mechanism that may be involved in repeated bout adaptation is the ability of heat shock proteins (HSP) to stabilise damaged structural proteins and thus protect against protein degradation. In this context, a stress-inducible member of the HSP70 family (also referred to as HSP72) has been demonstrated to function as a molecular chaperone overseeing the proper folding of denatured proteins and thereby facilitating the restoration of such proteins (Black & Subjeck 1991, Kiang et al. 1998, Liu et al. 2006). In addition, small HSP´s, (sHSP) like HSP27 and αβ-crystallin, are thought to bind directly to specific structural proteins in the sarcomers and protect them against denaturation (Koh & Escobedo 2004, Koh 2002). Single-bouts of eccentric exercise or running exercise have both been observed to elicit an acute increase in the protein expression of HSP27 and/or HSP70 (Feasson et al. 2002, Morton et al. 2006, Thompson et al. 2001). However, direct comparisons between isolated concentric versus isolated eccentric exercise have not been conducted. In addition, a study by Thompson and co-workers (2002) showed no differences in HSP70 and HSP27 expression responses to repeated eccentric exercise (Thompson et al. 2002). This does

not, however, rule out a role for HSP system components in repeated bout adaptation, since the adaptive responses of HSP components may also be evaluated as alterations in translocation, i.e. the movements of HSPs from one cellular compartment to another. To date, no studies have investigated the effect of repeated eccentric exercise on HSP translocation. This might however provide important information on the mechanisms involved in the immediate response to muscle damaging exercise and the adaptational events involved in the ability to sustain repeated muscle damaging exercise. Accordingly, in a recent study, we have observed the content of HSP27 and αβ-crystallin protein to be decreased in the cytosol and increased in cytoskeleton in response to single-bout eccentric exercise (Paulsen et al. 2007). This strongly indicates that a HSP translocation response is initiated to protect sarcomeric proteins that are damaged from eccentric exercise and suggests that such a translocation response is altered when a muscle is re-exposed to eccentric exercise of similar magnitude.

The purpose of the present study was therefore to investigate the exercise-induced changes in muscle HSP70, HSP27 and αβ-crystallin. More specifically, we wanted; (1) to compare the HSP protein contents in cytosolic and cytoskeletal muscle cell fractions and the HSP gene expression responses to single-bout concentric and single-bout eccentric exercise in human skeletal muscle, and (2) to investigate if such a HSP system response exhibited a repeated bout adaptation. Our hypothesis was to observe an upregulated HSP response to single-bout eccentric exercise, but not to concentric exercise and an attenuated response to the repeated eccentric exercise bout compared to the initial eccentric exercise bout**.**

MATERIALS AND METHODS

Subjects

Twenty healthy male subjects (mean ± standard deviation; age: 23.8 ± 2.8 years; weight: 77.8 ± 7.0 kg; height 182 ± 8 cm) were included in the study and were randomly assigned to the exercise-intervention (14 subjects) and to the control-intervention (6 subjects), as previously described (Vissing et al. 2008). Subjects had not been engaged in regular resistance training of the lower body or sports characterized by moderate to larger magnitudes of eccentric exercise modality for six months prior to inclusion in the study. The subjects were instructed not to change their habitual activity throughout the involvement in the study, except they were instructed to refrain from physical activity in the days prior to single-bout exercise or control trial.

All subjects received detailed information of the purpose and the possible risks of procedures and gave their written consent to participate. The study was approved by the Danish Ethical Committee of Aarhus (j. nr 20040159) and carried out in accordance with the Declarations of Helsinki.

Exercise protocol

The exercise groups performed a single bout of 30 minutes of bench-stepping at 60 steps per minute at a predetermined step height of 110% of the lower leg length, as previously described (Vissing et al. 2008, Newham et al. 1983). Stepping up with one leg entails muscles involved in knee extension to work concentrically, Stepping down with the same leg first then entails the same muscles of the opposite leg to work

eccentrically. Eight weeks after the first single bout, the subjects of the exercise group repeated the exercise bout following an identical protocol. Thus, four different exercise interventions were conducted, namely first and second bout concentric and first and second bout eccentric, respectively. Subjects performed concentric and eccentric work with the same legs in both bouts. Using this exercise protocol, the work done during the exercise can be determined using the mass lifted (body mass or body mass + 10 kg), the step height (110% of lower leg length - on average 58 cm) and the gravitational constant. Using the average values the step group performed 443 J of concentric work during each step cycle. An identical amount of eccentric work was performed during the down-stepping phase. Although the work is essentially performed by gravity, the muscles must contract to resist this force in order to produce a controlled slow down-stepping motion. Therefore the overall mechanical load on the muscles can be considered to be the same in the eccentric and concentric leg during the step-exercise. Subjects in the control group were tested as described below, but they performed no exercise and test procedures were not repeated after 8 weeks for this group.

Measurements of muscle force/pain assessment

Isometric maximal voluntary contraction (MVC) forces were measured as previously described (Overgaard et al. 2004). Before the first test, subjects were familiarized with the strength testing protocol. During the actual test, three trials were performed with verbal encouragement, and the best result was used for further analysis (Edwards et al. 1977). Subjective feeling of pain in the quadriceps muscle of concentrically and eccentrically working legs was assessed on a visual analogue scale (VAS-score) during rising from and sitting in a chair, as elsewhere described (Arendt-Nielsen

2002). MVC and VAS-score were measured prior to and at 3 hours, 24 hours, 2 days, 3 days, 5 days and 7 days after each bout (see figure 1). In addition MVC was measured 7 days prior to each bout of step exercise.

Blood sample analysis

Ten ml of blood were drawn from an antecubital vein prior to and at 3 hours, 24 hours, 2 days, 3 days, 5 days and 7 days after each bout (see figure 1.). Blood samples were immediately centrifuged and plasma fractions were stored at -20° until further analysis. Plasma samples were analysed for creatine kinase and myoglobin using a commercial kit applied in a multi-analyser system (COBAS Integra 700, Hoffmann-La Roche Ltd., Basel, Switzerland).

Biopsy sampling

Biopsies were obtained from m. vastus lateralis using a conchotome as described previously (Dietrichson et al. 1987) one week prior to each bout and at 3 hours, 24 hours and 7 days post exercise by procedures previously described (Vissing et al. 2008). Following the first bout, biopsies were obtained from both concentrically and eccentrically working legs, while after the second bout, biopsies were only obtained from the eccentrically exercised leg (see figure 1.). Biopsies from the control group were obtained from one predetermined leg. Following biopsy sampling the tissue was immediately frozen in liquid nitrogen and stored at -80° until further analysis.

mRNA isolation and Northern blotting

RNA was extracted using the guanidinium thiocyanate-phenol-chloroform extraction method previously described by Chomczynski and Sacchi (Chomczynski & Sacchi 1987). RNA concentrations were quantified and loaded as 200 ng per well on a denaturing formaldehyde agarose gel for separation and mRNA was quantified by Northern blotting using cloned PCR products for probe generation as previously described (Vissing et al. 2008, Jonsdottir et al. 2000) for heat shock protein 70 (HSP70), small heat shock proteins (sHSP) HSP27 and αβ-crystallin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) based on human muscle cDNA using Accuprime Taq PCR (Invitrogen) and specific primers (Table 1). The 28S rRNA probe was generated by end-tagging a 28S complementary oligo (Table 1) as previously described (Vissing et al. 2008). Probes were then added to the membranes and washed at high stringency conditions followed by exposure on phosphor screens as previously described (Vissing et al. 2008). Signals were captured by a phosphoimager and quantified using QuantityOne (Biorad) software.

Protein isolation and Western blotting

Muscle biopsies (10-30 mg) from ten of the fourteen exercise subjects and for the six control subjects were homogenized, and subcellularly fractionated in accordance with the ProteoExtract® Subcellular Proteome Extraction Kit (Merckbiosciences, catalogue #539790) as previously described (Paulsen et al. 2007). Protein content of cytosolic and cytoskeletal fractions was then determined using the RC/DC kit (Bio-Rad catalogue #500-0121). HSP27 and HSP70 protein levels in the cytosolic and cytoskeletal fractions were analyzed by Western blotting. However, since we wanted to compare all the treatments for each individual exercise subject for each fraction on the same

gel/membrane, we chose to load the gel with only pre, 3H and 7D time points for either cytosolic or cytoskeletal fractions for all treatments, as all four time points for each exercise treatment could not be loaded on the applied gels. Thus, the 24H time point was not included in protein analysis. For control subjects all four time points were loaded for both the cytosolic and the cytoskeletal fraction. Equal amounts of protein (15μg/well) were separated on NuPage gels (14% gradient gels, catalogue # NPO0321BOX) for 35 min at 200 volts and transferred to PVDF membranes for 60 min at 30 volts in NuPage® Transfer buffer. After an overnight block and a subsequent wash, membranes were incubated for 2h with a primary antibody against HSP27 (dilution 1:4000, StressGen SPA-800) and HSP70 (dilution 1:4000, StressGen SPA-810). The HSP70 antibody recognises inducible HSP70 (also referred to as HSP72) and does not cross-react with constitutively expressed HSP70 (also referred to as HSC73) (Black & Subjeck 1991). Blots were washed and then incubated with a secondary antibody conjugated to horseradish peroxidase (1:750; Pierce). After a final wash protein content was detected with chemiluminescence Super Signal West Dura (Pierce catalogue #. 34076**)** and digital images using a Kodak image station. Net signal intensity was measured as the sum intensity of the band area subtracted the sum intensity of a nearby representative background area using the Kodak 1D software (version 3.6.1, Kodak). GAPDH (Abcam, ab9484) and desmin (Dako, DE-R-11) were measured to ensure equal loading in cytosolic and cytoskeletal fractions, respectively, with no differences in expression found. Initially, representative samples were compared to a standard curve with recombinant protein and the HSP content in the samples were well within the linear range of the standar curve established. Blots are shown for HSP27, HSP70, GAPDH and Desmin for one representative exercise subject in figure 2.

Statistical analyses

Data for soreness, strength and plasma markers were subjected to two-way ANOVA for intervention vs. time followed by post hoc analysis (Student Newman-Keuls test) as previously described (Vissing et al. 2008), with first and second bout concentric and first and second bout eccentric exercise constituting the four different interventions. For mRNA and protein data, only three different interventions were analysed since biopsies were not harvested from scond bout concentric exercise. All RNA data were normalized to 28S rRNA. 28S-normalized mRNA data-values within each intervention were then normalized to their respective PRE values (obtained one week prior to each exercise bout), log-transformed and tested by two-way ANOVA for intervention x time for an effect of intervention, time or both followed by post hoc analysis (Student Newman-Keuls test). Protein data were treated the same way, except they were not normalised to an internal control protein. Changes in basal mRNA and HSP protein levels were tested with a t-test. All statistics were done using SigmaStat and Microsoft Excel. Data for controls were subjected to one-way ANOVA for time to test if time affected the signal in any sample. If an effect for time was seen, post hoc analysis (Student Newman-Keuls test) was performed. mRNA and protein data are presented as geometric means ± back-transformed standard error. For all post hoc tests $p<0.05$ was considered significant.

RESULTS

The data on muscle strength, muscle soreness, plasma myoglobin and plasma creatine kinase for all fourteen exercise subjects are previously presented in more detail

(Vissing et al. 2008). The results of these measures for the ten out of fourteen exercise subjects specifically included in protein analysis of the current study did not differ from these previously reported results, summarised in brief below. For more detailed information and graphs of these results we refer to Vissing et al. (2008) (Vissing et al. 2008).

Markers of muscle damage

For MVC, an intervention x time interaction was observed ($p<0.001$) with a 15-17% reduction 1-2 days after first bout eccentric exercise. This differed from what was observed with both concentric and repeated eccentric exercise, where there was virtually no noticeable reduction ($p<0.05$). For muscle soreness, an intervention x time interaction was observed ($p<0.001$), with perceived soreness 1-2 days after first bout eccentric exercise being more than twice that of concentric exercise and 30-40% higher than that experienced after repeated eccentric exercise ($p<0.05$). For plasma myoglobin and plasma creatine kinase, an intervention x time interaction was observed ($p<0.001$), with up to ~ 100-fold and ~ 1000-fold increases, respectively, on day 3 and 4 after the first bout of eccentric exercise, compared to after repeated exercise ($p<0.05$). No changes in MVC, muscle soreness and plasma markers were observed in control subjects.

HSP protein content in cytosolic and cytoskeletal fractions

An example of the results from western blotting is shown in figure 2. No significant changes in HSP70 cytosolic content occurred in either the concentric or the eccentric exercise treatments (figure 3-a). An overall time effect ($p<0.001$) was observed,

however, in HSP70 cytoskeletal content and a 2.4-fold increase was observed 7 days after the first bout of eccentric exercise. This increase observed in the eccentric leg, after the first bout, was not significantly different from either the concentric or second bout eccentric exercise (figure 3-b). No changes in HSP70 cytosolic and HSP70 cytoskeletal content were observed in control subjects (results not shown). No changes in HSP70 basal levels (pre first bout versus pre second bout) were observed in either cell fraction (results not shown).

No changes in HSP27 cytosolic content occurred during either concentric or eccentric exercise treatments (figure 4-a). An intervention x time interaction was observed in HSP27 cytoskeletal content ($p<0.001$), with a 9.8-fold increase in HSP27 cytoskeletal content observed 3 hours after the first bout of eccentric exercise which was significantly different from what was observed for both concentric exercise, where no change was noted, and second bout eccentric exercise, where only a 3.9-fold increase was observed (4-b). No changes in HSP27 cytosolic and cytoskeletal content were observed in control subjects (results not shown). No changes in HSP27 basal levels (pre first bout versus pre second bout) were observed in either cell fraction (results not shown).

HSP mRNA expression

For HSP70 mRNA an intervention x time interaction was observed ($p<0.001$). During the time-course of sampling post exercise, HSP70 was upregulated in response to eccentric exercise when compared to the pre-value (figure 5a). First bout eccentric exercise induced a 8.9-fold upregulation at the 3 hour time point ($p<0.001$) declining to a 1.8-fold upregulation ($p<0.001$) at the 24 hour time point after which it declined

to baseline level at 7 days post exercise. A tendency ($p=0.061$) to an attenuated upregulation (only 4.9-fold) was observed at the 3 hour time point after the second compared to the first eccentric exercise bout. No changes were observed in response to the concentric and control treatments – see figure 5a.

HSP27 and αβ-crystallin mRNA exhibited almost identical expression patterns and for both targets, an intervention x time interaction was observed ($p<0.001$) (figure 5b and 5c). During the time-course of sampling post exercise, HSP27 and αβ-crystallin were upregulated in response to eccentric exercise when compared to the pre-value. First bout eccentric exercise induced a 2.9-3.2-fold upregulation ($p<0.001$) at 3 and 24 hour time points for both HSP27 and αβ-crystallin, after which expression declined to baseline level at 7 days post exercise. After the second bout of eccentric exercise, the upregulation at the 3 hour time point was attenuated to 1.8-fold for αβ-crystallin ($p<0.05$) and a tendency to a similar level of attenuation was observed for HSP27, whereas the level of upregulation for both mRNA targets did not differ from first bout eccentric exercise at the 24 hour time point. No changes were observed in response to concentric and control treatments – see figure 5b-c. No changes in basal levels (pre first bout versus pre second bout) were observed (results not shown).

DISCUSSION

This study provides an insight into the effects of concentric versus repeated eccentric exercise on HSP distribution in different cellular compartments.

The main findings of the study were (1) that HSP cytoskeletal contents and mRNA expression levels were upregulated in response to damaging eccentric muscle

exercise, but not to concentric exercise, and (2) that this HSP response to eccentric exercise was attenuated concurrently with attenuation in muscle damage when eccentric exercise was repeated eight weeks later. Collectively, these results support the involvement of HSP system components in the immediate protection of sarcomeric proteins in a dose-response dependent manner determined by the level of muscle damage.

HSP translocation response to single-bout and repeated eccentric exercise

During the time course of recovery from the first exercise bout we observed an early and profound increase in the cytoskeletal content of HSP27 exclusively in the eccentrically working leg. For inducible HSP70, we observed a later overall upregulation that was seemingly greater in response to eccentric exercise, although it was not significantly different from that observed following concentric exercise. The time course of increased cytoskeletal HSP27 and HSP70 protein content is in line with our previous findings for single bout eccentric exercise (Paulsen et al. 2007) and suggests that HSP´s are translocated from the cytosol to the cytoskeleton to protect myofibrillar proteins against muscle damage induced from eccentric exercise (Paulsen et al. 2007, Koh & Escobedo 2004).

Contrary to our previous findings, this increase in cytoskeletal HSP was not accompanied by a concurrent decrease in HSP27 cytosolic content (Paulsen et al. 2007). However, our previous exercise protocol provided a much more intense exercise stimulus and produced a more profound muscle force loss and increase in HSP27 cytoskeletal content than the present one, and still just a relatively small decrease in HSP27 cytosolic content (Paulsen et al. 2007). Thus, it seems likely, that

only a smaller part of a larger cytosolic pool of HSP27 is actually binding to the cytoskeletal proteins in a dose-response dependent manner and that a concurrent downregulation in the cytosol may be below the limit of detection in the present study. In our previous studies on single-bout eccentric exercise, αβ-crystallin was observed to react in exactly the same manner as HSP27 (Paulsen et al. 2007). The current results on mRNA expression also provide further support for the notion, that HSP27 and αβ-crystallin undergo similar regulation. Therefore, for practical reasons, protein analysis of αβ-crystallin was omitted from the current study.

One previous study by Thompson et al (2002) investigated the protein expression responses of HSP70 and HSP27 to repeated isolated eccentric exercise (Thompson et al. 2002). In this study, both HSP70 and HSP27 were upregulated in response to eccentric exercise, but no differences were found in induction for either of the two HSP´s between a first and a repeated eccentric exercise bout interspaced by four weeks of recovery (Thompson et al. 2002). However, the analysis performed by Thompson and co-workers relied on crude homogenate. Thus, it does not provide information on distributional changes of HSP70 and HSP27 within the exposed muscle cells. Here we provide evidence that the HSP27 cytoskeletal content is substantially attenuated early in the recovery phase upon repeated eccentric exercise, when compared to first bout eccentric exercise. This finding may indicate that the necessity for HSP27 is diminished due to improved resistance of cytoskeletal components against degradation. In accordance, it has been suggested that muscle can adapt to better resist muscle damage by increasing the number of sarcomers in series (Lynn & Morgan 1994, Lynn et al. 1998, Brockett et al. 2001). We therefore

speculate that adaptation to first bout eccentric exercise at least partly occurs as a result of an increased capability of the sarcomers to withstand repeated exposure to exercise of similar modality and strain. Thus, since less myofibrillar damage occurs on repeated exposure, less HSP27-mediated protection is necessary and in consequense less translocation of HSP27 from cytosol to cytoskeleton takes place.
A similar repeated bout effect on HSP70 translocation was not observed. Had analysis of the 24 hour time point been conducted, our ability to evaluate HSP70 translocation might have been somewhat improved. On the other hand, our previous studies on single-bout eccentric exercise indicate that HSP70 translocation reaches peak increase at 24 hours and remains level between 24-175 hours (Paulsen et al. 2007), which supports our 7 day time point as a representative time point.
In the study by Thompson et al. (2002), reduced basal levels of HSP70 and HSP27 protein expression were observed prior to the repeated eccentric exercise bout compared to the first bout of eccentric exercise (Thompson et al. 2002), which supports the hypothesis of a decreased necessity for HSPs to protect against myofibrillar damage due adaptational events leading to improved resistance of the sarcomeric components. Since our analysis were performed on fractionated tissue, we were unable to make a similar direct comparison on the total basal levels of HSP70 and HSP27 protein. Instead, working on the assumption that most HSP70 and HSP27 exists in the cytosol in resting muscle, we compared the HSP expression levels of the cytosol of the repeated bout relative to the first bout, but found no differences for either HSP target. Furthermore, basal levels of mRNA expression were not observed to differ between bouts (see below). Thus, in contrast to Thompson et al. (2002) , our

data do not support that changes in pre-exercise HSP levels contribute to the repeated bout effect (Thompson et al. 2002).

HSP gene expression response to single-bout and repeated eccentric exercise

Our mRNA quantitative analysis allowed us to include the 24 hour time point and also, the sHSP αβ-crystallin was added to the analysis of HSP70 and HSP27.

No changes in basal levels were observed between exercise bouts. Similar patterns of upregulation for HSP27 and αβ-crystallin during the immediate recovery from single-bout eccentric exercise, were observed, and HSP70 was also upregulated, but to a greater extent and peaking earlier than the sHSP´s. In this context, it should be emphasized, that several different genes exist for HSP70 (Liu et al. 2006). Our probe only recognized HSP70A (HSP70-1) and HSP70C (HSP70-2), collectively referred to as HSP72. These isoforms have been previously reported to respond to exercise (Liu et al. 2004, Willoughby et al. 2002). Thompson and co-workers measured HSP70A, HSP70B, and HSP70C and found HSP70C to be increased in biceps brachii muscle after maximal eccentric exercise, and HSP70B to be increased in vastus lateralis muscle after downhill running (Thompson et al. 2003). Thus, regulation of the different HSP70 genes certainly requires further investigation, as different isoforms might respond differently to single-bout and repeated eccentric exercise.

Interestingly, all three HSP targets exhibited an attenuated level of upregulation three hours after the repeated eccentric exercise bout compared to the first eccentric exercise bout (although there was only borderline significance for HSP70 and HSP27), and yet, similar expression levels 24 hours after both the first and the repeated eccentric bouts. Thus, HSP gene expression seems to be somewhat

decelerated during the early hours of recovery after repeated muscle damaging exercise. We speculate that this decelerated expression supports the notion of a decreased necessity for HSP-mediated protection of myofibrillar proteins that have adapted to withstand repeated eccentric exercise. An alternative explanation could be that the early HSP expression changes are related primarily to developing an increased defence towards muscle damage, whereas the later expression changes are related primarily to remodelling processes, (Liu et al. 2006). However, our data provide no clear explanation for the equalisation between bouts in gene expression levels later in the recovery phase and we are therefore unable to conclude strongly on the bearings of these findings.

Concentric exercise and non-exercising control intervention

Using the described step protocol, we were able to compare directly the effects of isolated concentric versus isolated eccentric exercise. Muscle force loss and muscle soreness were of a higher magnitude in the eccentrically working leg compared with the concentrically working leg. However, no changes were observed in the concentrically working leg for cytosolic and cytoskeletal HSP protein content. Concentrically based endurance exercise has previously been demonstrated to be able to elicit an increased expression of HSP70 (Morton et al. 2006, Febbraio & Koukoulas 2000, Vissing et al. 2005). For instance, in the studies by Morton et al (2006) minor increases in HSP expression were observed in response to single-bout non-damaging running exercise, suggesting that concentric exercise may to a lesser extent elicit a HSP response (Morton et al. 2006). However, unlike the aforementioned studies, our step protocol involved exercise of shorter duration and

with less demand on oxidative metabolism. Consequently, it is likely that our protocol did not impose a similar rise in oxidative stress and/or total muscle glycogen expenditure, both of which have been related to HSP-elicitations (Febbraio et al. 2002, Khassaf et al. 2003). In previous studies, for example, it has been demonstrated that glycogen depletion by the step protocol amounted to approximately 20% and exclusively in the concentrically working leg (Fredsted et al. 2008). Finally, even level running exercise will inevitably involve an eccentric work, which makes it difficult to distinguish concentric from eccentric exercise modalities using running protocols. Our step protocol may therefore be regarded as a more valid model than running exercise for studying HSP responses to concentric exercise and/or for comparison between eccentric and concentric exercise.

Driving factors for regulation of HSP system components

We investigated possible correlations between the measured parameters in question, but we found no strong relationship between factors like e.g. strength reduction, muscle soreness and mRNA expression or HSP cytoskeletal content and we did not perform Electron microscopy-analysis. We can therefore only speculate on the exact driving factor that elicits the HSP response. Still, we find it feasible that ultrastructural damage induced by eccentric exercise is the driving factor behind the HSP responses we observe.

From our present study, evidence is provided, that the eccentric exercise modality is a stronger mediator of HSP responses than the concentric exercise modality. As previously mentioned, this response is likely to be primed by the muscle damage inflicted by this exercise modality, whereas concentric exercise does not, or only to a

minor degree inflict such muscle damage. In regard to muscle damage, muscle biopsy sampling might in itself be thought to initiate an HSP response and, in accordance, it has been previously demonstrated, that exercise-induced expression of e.g. HSP70 and αβ-crystallin can be triggered by invasive procedures like biopsy sampling (Vissing et al. 2005, Fluck et al. 2005, Malm 2001). We therefore added a non-exercising control group to our study. As we did not observe any changes in protein and gene expression levels compared to basal levels in the control group for either of the HSP targets that were the focus of our investigation, we are therefore more confident that what we observed was, in fact, a result of the exercise intervention in question.

In summary, our protocol established a repeated bout effect from eccentric exercise as evidenced by the reduction in muscle strength, muscle soreness and plasma markers for muscle damage. HSP translocation and expression responded exclusively to eccentric exercise and the overall HSP responses were attenuated when eccentric exercise was repeated. Thus, the extent of the HSP response seems to be determined by the level of muscle damage inflicted by the exercise bout. Our data support a role for of increased translocation and increased expression and, thus add to the understanding of the mechanisms involved in the recovery from and adaptation to muscle-damaging exercise.

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CONFLICT OF INTEREST

There is no conflict of interest.

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TABLES

Table 1. PCR and oligo probe information

Target	**Probe sequence**	**mRNA accession #**
HSP70	Sense ATGAAGAGCGCCGTGGAGGAT Antisense TTGGAAAGGCCCCTAATCTA	NM_005345
HSP27	Sense TCTCGGAGATCCGGCACACT Antisense GGCATCCAGGCTAAGGCTTT	NM_001540
αβ-crystallin	Sense GGAAAACATGAAGAGCGCCA Antisense TGGGCCTGCCCTTAGCATTA	NM_001885
GAPDH	Sense GAACATCATCCCTGCCTCTACT Antisense GTCTACATGGCAACTGTGAGGA	NM_002046
28S	TCGCCGTTACTGAGGGAATCCTGGTTAGTTTCTTT*	M11167

*The 28S sequence is not a primer sequence but the probe itself.

LEGENDS

Figure 1. Experimental protocol. Untrained male volunteer subjects performed a single bout (1. bout) of 30 min step exercise (E), which was repeated after 8 weeks (2. bout) and compared to non-exercising control subjects (÷ E). Isometric strength (MVC), muscle soreness (VAS), venous blood and muscles biopsies were sampled at designated time-points pre and post exercise except VAS-score and blood sampling which were not performed 7 days prior to each bout.

Figure 2. Western blots from one representative exercise subject. Blots are shown for pre, 3h and 7d time points for 1. bout concentric (conc) and eccentric (ecc 1) exercise and for 2. bout eccentric exercise (ecc 2). HSP70 and HSP27 are shown in cytosolic and cytoskeletal fraction with GAPDH and desmin as loading controls for both fractions, respectively.

Figure 3. HSP70 cytosolic and cytoskeletal protein content. Changes in HSP70 cytosolic and cytoskeletal protein content are shown in response to concentric (Conc), eccentric bout 1 (ECC1) or eccentric bout 2 (ECC2). Data are presented as geometric mean +/- back-transformed SEM shown as fold changes from the individual pre values of the specific leg and bout. Results of two-way ANOVA (intervention, time and/or intervention x time interaction) are shown in upper left corner of each figure. * denotes different from PRE, $p<0.05$.

Figure 4. HSP27 cytosolic and cytoskeletal protein content. Changes in HSP27 cytosolic and cytoskeletal protein content are shown in response to concentric (Conc), eccentric bout 1 (ECC1) or eccentric bout 2 (ECC2). Data are presented as geometric mean +/- back-transformed SEM shown as fold changes from the individual pre values of the specific leg and bout. Results of two-way ANOVA (intervention, time and/or intervention x time interaction) are shown in upper left corner of each figure. * denotes different from PRE, $p<0.05$. # denotes different from Ecc1, $p<0.05$

Figure 5. mRNA expression. mRNA response to concentric (Conc), eccentric bout 1 (ECC1), eccentric bout 2 (ECC2) or control treatment is shown for HSP70 (a), for HSP27 (b) and for αβ-crystallin (c). Data are normalised to 28S rRNA and presented as geometric mean +/- back-transformed SEM shown as fold changes from the individual pre values of the specific leg and bout. * denotes different from PRE, # denotes different from ECC1. Results of two-way ANOVA (intervention, time and/or intervention x time interaction) are shown in upper left corner of each figure.

Figure 1

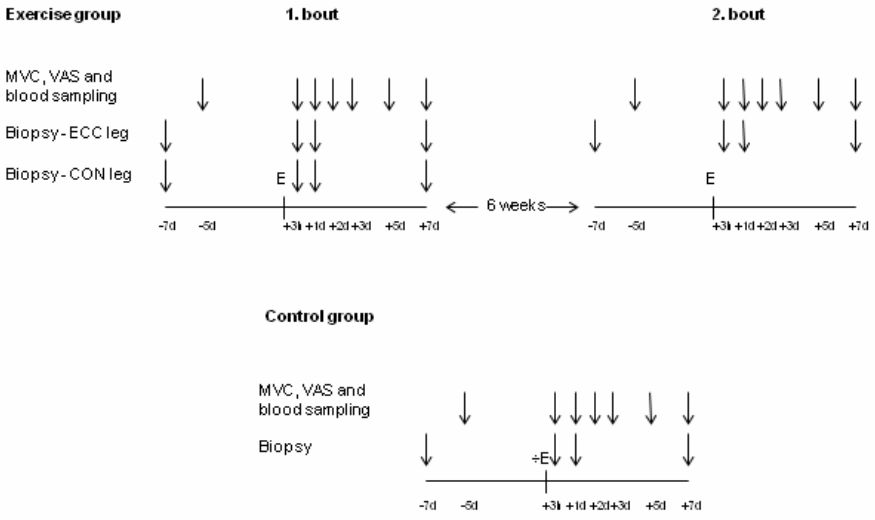

Exercise group
1. bout
2. bout
MVC, VAS and blood sampling
Biopsy - ECC leg
Biopsy - CON leg
E
E
6 weeks
-7d
-5d
+3h
+1d
+2d
+3d
+5d
+7d
Control group
MVC, VAS and blood sampling
Biopsy
E

Figure 2

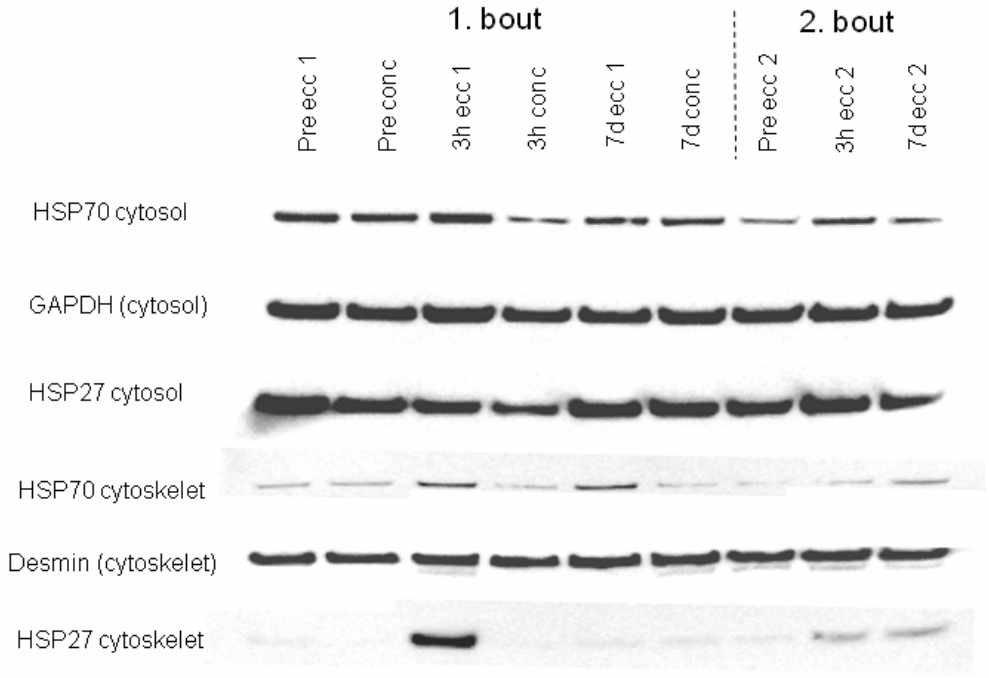

1. bout
2. bout
Pre ecc 1
Pre conc
3h ecc 1
3h conc
7d ecc 1
7d conc
Pre ecc 2
3h ecc 2
7d ecc 2
HSP70 cytosol
GAPDH (cytosol)
HSP27 cytosol
HSP70 cytoskelet
Desmin (cytoskelet)
HSP27 cytoskelet

Figure 3

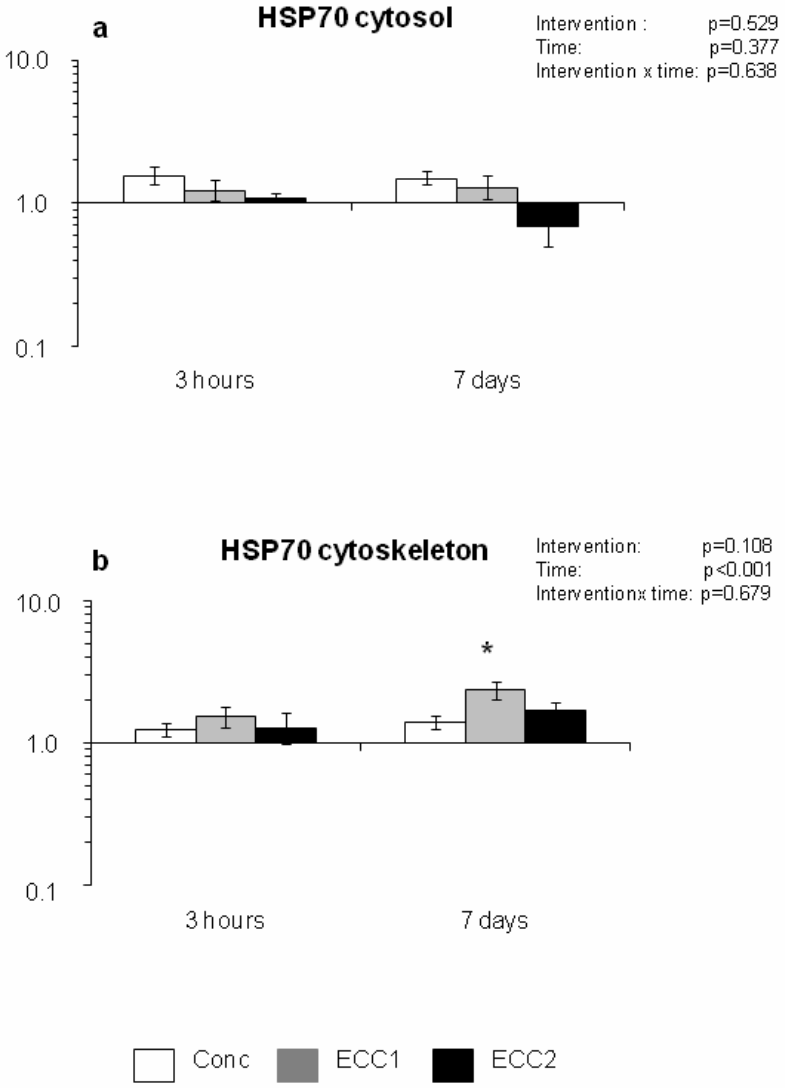

a
HSP70 cytosol
Intervention : p=0.529
Time: p=0.377
Intervention x time: p=0.638
10.0
1.0
0.1
3 hours
7 days
b
HSP70 cytoskeleton
Intervention: p=0.108
Time: p<0.001
Intervention x time: p=0.679
*
10.0
1.0
0.1
3 hours
7 days
Conc
ECC1
ECC2

Figure 4

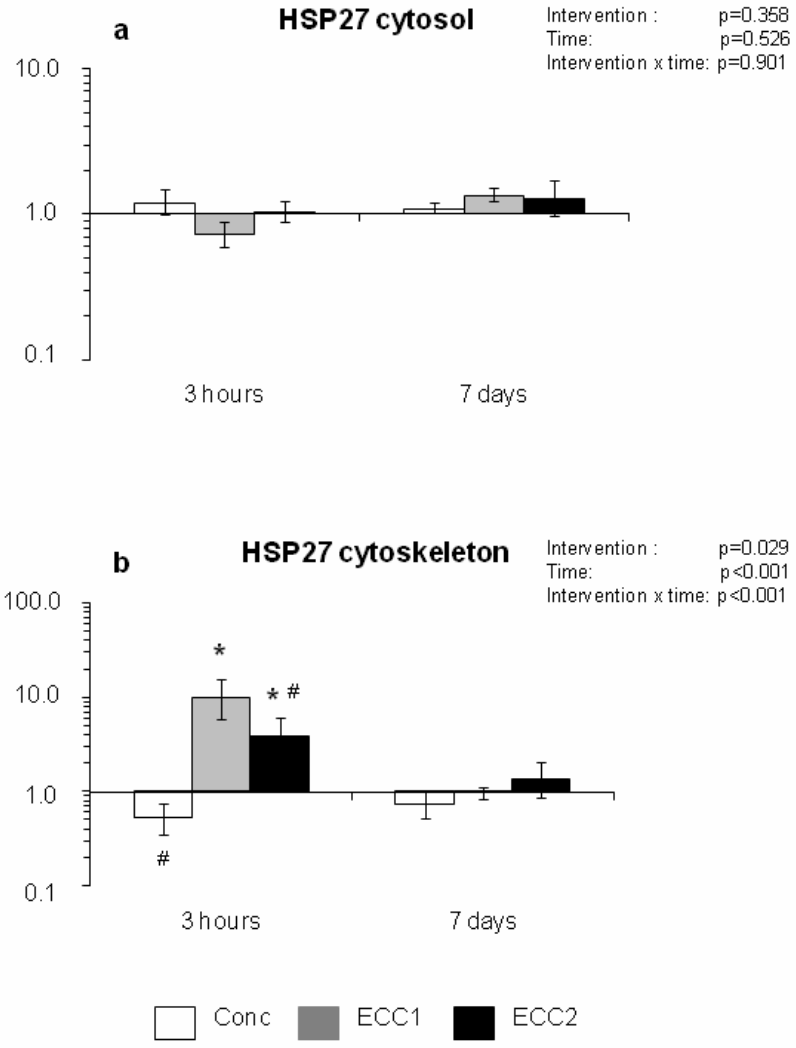
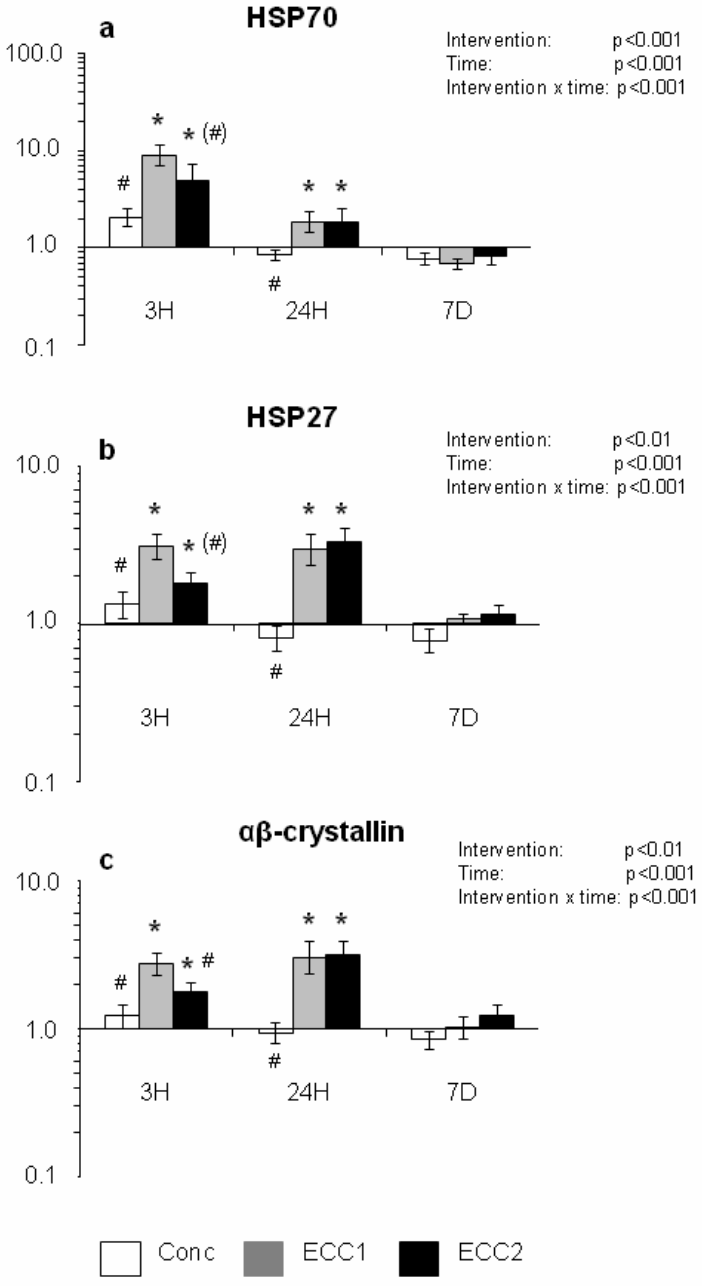

a
HSP27 cytosol
Intervention : p=0.358
Time: p=0.526
Intervention x time: p=0.901
10.0
1.0
0.1
3 hours
7 days
b
HSP27 cytoskeleton
Intervention : p=0.029
Time: p<0.001
Intervention x time: p<0.001
100.0
10.0
1.0
0.1
*
* #
#
3 hours
7 days
Conc
ECC1
ECC2

Figure 5


a
HSP70
Intervention: p<0.001
Time: p<0.001
Intervention x time: p<0.001
100.0
10.0
1.0
0.1
3H
24H
7D
b
HSP27
Intervention: p<0.01
Time: p<0.001
Intervention x time: p<0.001
10.0
1.0
0.1
3H
24H
7D
c
αβ-crystallin
Intervention: p<0.01
Time: p<0.001
Intervention x time: p<0.001
10.0
1.0
0.1
3H
24H
7D
Conc
ECC1
ECC2