Immunohistochemical changes in the expression of HSP27 in exercised human vastus lateralis muscle.

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HSP27, resistance and endurance exercise
Abstract

**Aim:** The role of HSP27 in the adaptive process of skeletal muscle to exercise, especially in humans, is not well understood. The objective of this study was to investigate immunohistochemical changes in HSP27 expression in human vastus lateralis muscle following resistance and endurance exercises.

**Methods:** Two different exercise protocols were used: (1) one-leg ergometer cycling (EC, n=6) consisting of two 30 minute bouts at 40 and 75% of peak oxygen uptake, respectively, and (2) leg extension resistance exercise (RE, n=9) including 10 sets of 8 repetitions at a load corresponding to 70% of one maximal repetition (1 RM). Immunohistochemistry using specific monoclonal antibodies was used to determine the location of HSP27 protein in muscle biopsies from human vastus lateralis.

**Results:** Our results show that RE, but not EC, induced a significant appearance of scattered accumulations of HSP27 protein in muscle fibres from 5 out of 9 subjects. The number of fibres with accumulation of HSP27 in RE ranged from 0 to 32% with a mean of 6.3% of the total number of fibres.

**Conclusion:** We conclude that this rapid HSP27 protein relocation after RE is an important player in the cellular remodelling of human muscle fibres in response to exercise involving high-force contractions, but not in response to endurance exercises.

**Key Words:** Stress response, heat shock protein, endurance and resistance exercise, fibre type, adaptation
Skeletal muscle fibres are constantly exposed to various forms and amounts of stress during normal daily activities and in some populations regularly to more extreme physiological conditions. In response to exercise training, the type of stress put on muscle fibres varies from being mainly mechanical, due to forceful contractions during strength exercises, to primarily metabolic during endurance activities with high demands on oxygen delivery and energy transfer. The exposure to different forms of stress may explain specific muscular adaptations to exercise. Although the structural and metabolic changes that occur in response to different types of exercise are well characterized, less information is available on the molecular pathways behind the adaptive events to strength and endurance exercises.

Heat shock proteins (HSPs) represent a family of stress proteins activated in response to various forms of stress such as increased temperature (Kelley et al. 1980; Kelley and Schlesinger 1978), hypoxia (Benjamin et al. 1990; Mestril et al. 1994), acidosis (Nishimura and Dwyer 1995; Poso et al. 2002), reduced glucose availability (Lanks 1983; Sciandra and Subjeck 1983) and sheer mechanical stress (Knowlton et al. 1991). HSP family members are generally named after their molecular weight, and the different HSPs can facilitate appropriate cellular adaptation as they play a fundamental role in protein synthesis (Beckmann et al. 1990), degradation (Ciechanover et al. 2000), stabilization (Jakob et al. 1993; Lutsch et al. 1997) and are also involved in intracellular signalling (Helmbrecht et al. 2000).

Locke et al (1990) were the first to show that exercise is a sufficient stimulus to induce a significant HSP response in mammalian cells and tissues, including skeletal muscle. Subsequently it was shown that endurance exercise (Febbraio and Koukoulas 2000; Khassaf et al. 2001; Liu et al. 2000; Liu et al. 1999; Puntschart et al. 1996) as well as exercises demanding high muscular force (Liu et al. 2004; Paulsen et al. 2007; Thompson et al. 2002; Thompson et al. 2003; Thompson and Scordilis 1994; Thompson et al. 2001) can induce a
significant HSP response in human vastus lateralis muscle. HSP27 is a low molecular weight protein located in the myofibrillar compartment of the muscle fibre (Lutsch et al. 1997). Furthermore HSP27 is thought to be involved in the modulation of cytoskeletal structures, as it is engaged in the organization and stabilization of actin filaments. Therefore it is suggested that HSP27 is involved in an actin-based adaptive response of muscle fibres to new environmental conditions (Landry and Huot 1995; Lavoie et al. 1993; Mounier and Arrigo 2002).

The role of HSP27 in the adaptive process of skeletal muscle to exercise, especially in humans, is not well understood and has been the subject of relatively few investigations. Thompson et al. (2001, 2002) showed an up-regulation, 48 hours after exercise, of HSP27 protein level in skeletal muscle in response to high force eccentric exercises, but not after downhill treadmill running (Thompson et al. 2003). In contrast, Féasson et al (2002), using a downhill treadmill running protocol similar to that used by Thompson et al (2003), showed an increase in protein levels at 1 and 14 days after the exercise bout. More recently, Morton et al (2006) investigated the time course of HSP27 response following acute non-damaging treadmill running at an intensity close to the lactate threshold. Biopsies were sampled before exercise and at 24 h, 48 h, 72 h and 7 days after exercise. There were no changes in HSP27 protein level at any time point (Morton et al. 2006). In the above studies, protein level of HSP27 has been investigated quantitatively from muscle homogenates using Western blot analysis. The pattern of expression of HSP27 in human vastus lateralis muscle tissue and the exact location of the protein following exercise has to our knowledge only been addressed in one study (Paulsen et al. 2007). It was suggested that an exercise protocol consisting of 300 maximal eccentric contractions, inducing severe muscle soreness and loss of muscle force, is accompanied by an accumulation of HSP27 around myofibrils (Paulsen et al. 2007). This
supports the results previously seen in a study on lengthening contractions in murine skeletal muscle (Koh and Escobedo 2004).

Given the above discussion, the aim of the present study was to investigate the immunohistochemical expression of HSP27 in different structures of skeletal muscle including type I and type II muscle fibres. The study also aimed to further understand the involvement of HSP27 in the adaptive process of human vastus lateralis muscle to different forms of exercise. Immunohistochemical expression of HSP27 in vastus lateralis was studied in response to resistance and endurance exercises.
MATERIALS AND METHODS

Subjects

Two different exercise protocols were used in this study: one-leg ergometer cycling (EC, n=6) and resistance exercise (RE, n=9). Subject characteristics are shown in table 1. All subjects participating in the study were healthy and physically active male students. None of the subjects were engaged in any exercise training program, apart from their habitual physical activity. All experimental protocols and biopsy procedures were approved by regional ethics committees and the protocols complied with the standards set by the Declaration of Helsinki.

Experimental design

For the subjects performing EC, peak oxygen uptake (VO2 peak) for one-leg cycling was determined 1-2 weeks before the tests were undertaken (2.5 ± 0.6 l·min⁻¹). All subjects were familiarised with the one-leg exercise cycling protocol before the actual test. The cycle ergometers were obtained from Monark (Vansbro, Sweden), the ergometer used for one-leg cycling being equipped with an extra heavy wheel in order to create momentum for returning the pedal for subsequent pedalling movements. The pedalling rate was 60 rpm. Expired air was collected in Douglas bags and subsequently measured for volume in a balanced Tissot spirometer (Warren Collins, Braintree, MA, USA) and analysed for O₂ and CO₂ percentages using paramagnetic and infrared techniques, respectively (Beckman Applied Electrochemistry, Sunnyvale, CA, USA). On each of the two experimental days, 6–9 days apart, subjects performed one-leg cycle ergometry with their right leg, at one of two different exercise intensities, corresponding to 40 and 75% of the one-leg VO₂ peak respectively. The total duration of the one-leg cycle ergometry exercise bout was 30 minutes. The resting leg,
which served as a non-exercised control, was placed comfortably on a platform and the subjects were instructed to keep it relaxed during the exercise bout.

The RE consisted of leg extension resistance exercise at an intensity corresponding to 70% of 1 RM, and the exercise session included 10 sets of 8 repetitions. Before this session all subjects were familiarized with the exercise equipment, a commercial knee-extensor machine (Technogym, Gambettola, Italy). Seat depth, angle, and lever arm were carefully adjusted and positions were saved for later use. Determination of 1 RM was carried out after a low-intensity warm up on both a bicycle ergometer and the knee-extensor machine. During the 1 RM test, loads were increased gradually until the 1 RM was reached. Exercise was performed over a range of motion of 100 to 30 degrees knee angle, with 0 degrees being the fully extended position. Contractions were made at a self-selected pace with typically less than three seconds for each repetition, i.e. concentric and eccentric phases together. All eccentric contractions were performed fully controlled by the subjects, i.e. actively resisting the load while lowering the leg. Normalized strength was assessed using the allometric scaling of muscle strength to body weight for each subject according to the following formula: absolute strength × body mass$^{-2/3}$. (Folland et al. 2008).

Muscle biopsies

All muscle biopsies were taken from the mid-portion of vastus lateralis muscle under local anaesthesia. The samples were obtained using the Weil-Blakesley’s conchotome technique, immediately embedded in an embedding medium, frozen in isopentane cooled in liquid nitrogen and stored at -80°C until analysed. Muscle biopsies from both EC and RE were taken before and immediately after exercise.
Immunohistochemical staining on cross sections

Five μm thick muscle cross-sections were cut at about -20°C using a cryostat microtome (Leica CM1850, Leica Microsystems, Kista, Sweden). The sections were mounted on glass slides, air-dried at room temperature and then washed in phosphate-buffered saline (PBS) for 10 minutes. Thereafter sections were incubated for 20 minutes in normal blocking horse serum and then incubated overnight with the primary antibody. Two well-characterised monoclonal antibodies (mAb) were used: mAb A4951 (DSHB, Iowa, IA, USA), which strongly reacts against myosin heavy chain type I and mAb for HSP27 (NCL-HSP27, Novocastra, Newcastle upon Tyne, United Kingdom). Sections were then washed for 20 minutes in PBS and incubated for one hour with the diluted biotinylated horse anti-mouse secondary antibody (Vector BA-9200, Burlingame, CA, USA). For visualisation of primary antibodies Vectastain ABC (PK6100) reagent and DAB substrate kit (SK-4100) for peroxidise were used (Vector Laboratories, USA). The sections were visualised with light microscopy (Nikon Eclipse E400, Nikon Instruments, Amstelveen, The Netherlands) and images were acquired with a digital camera (SPOT Insight, Diagnostic Instruments, Sterling Heights, MI, USA) connected to the microscope.

Fibres showing cytoplasmic accumulations of HSP27, giving a granular appearance to the cell (fig 1), were counted as HSP27 positive fibres. Identification of positive and non-positive fibres was usually straightforward. The percentage of HSP27 positive fibres relative to the total number of fibres was calculated. For each biopsy an average of 249 fibres were analyzed.

PAS staining
Sections were fixed in Carnoy solution for 10 min, washed in distilled water and oxidized by periodic acid and then immersed in Schiff's reagent for 15 min. Sections are then counterstained in Ehrlich hematoxilin.

Statistics

Values are presented as mean, standard deviation and range. Wilcoxon’s signed rank test was used to analyze differences in the number of fibres showing cytoplasmic accumulations of HSP27 before and after the exercise protocols. The Spearman rank correlation was used to describe the relationship between two variables. The 0.05 level was used to indicate statistical significance.
Results

Immunohistochemistry using the HSP27 antibody in human vastus lateralis muscle showed no staining at the level of capillaries, connective tissue or the muscle fibre membrane. In biopsies sampled before as well as after the exercise, the cytoplasm of muscle fibres was slightly stained using the HSP27 antibody. Furthermore, we found that this homogeneous HSP27 staining was slightly stronger in type II compared to type I muscle fibres (fig 2). This fibre type pattern was seen in 13 out of 15 subjects whereas in the remaining two subjects there were no detectable difference in the staining intensity between type I and II fibres. In contrast to the light homogenous cytoplasmic staining, muscle fibres showing granular cytoplasmic accumulations of HSP27 appeared in biopsies taken immediately after completed resistance exercise, but not following endurance exercises (fig 3 A-D). These granular accumulations, where HSP27 tended to cluster in specific parts of the fibre, were never seen in biopsies taken before the exercise. HSP27 granular accumulations were seen in 5 out of 9 subjects in response to RE, and the number of fibres with HSP27 accumulation ranged from 0 to 32% with a mean of 6.3% of the total number of fibres (fig 4). All fibres within a muscle biopsy expressing these granular accumulations of HSP27 were identified as type II fibres.

In order to assess possible interaction between glycogen depletion and HSP27 response we analysed glycogen content before and after exercise in both EC and RE. Glycogen depletion was seen only in muscle biopsies taken after the EC protocol (fig 5 A-B). The RE protocol did not induce glycogen depletion in muscle fibres (fig 5 C-D). We have also assessed the relationship between muscle strength in subjects participating in RE and the HSP27 response. There was no significant correlation between normalized strength and the number of fibres showing cytoplasmic accumulations of HSP27 (r=-0.2, p=0.6).
Discussion

In the present study we have used immunohistochemistry on muscle cross-sections to characterize qualitative changes in HSP27 in human vastus lateralis muscle following two different exercise protocols including: 1) resistance exercise with heavy loads and 2) one-leg cycle exercise for 30 minutes at two different intensities. The main finding was the appearance of fibres containing granular accumulations of HSP27 immediately after the resistance exercise, but not after the endurance exercises. The cellular adaptations to exercise are commonly discussed in terms of increased or decreased protein levels. In this study, we show that the rapid HSP27 protein relocation within the muscle fibre is part of the acute adaptive process to strength exercises but not to endurance exercises.

The use of immunohistochemistry allowed us to show that a major early response to exercise can be protein relocation within the fibre. We propose that protein relocation is an early event in the adaptive process to exercise in humans, leading to a subsequent cascade of events eventually followed by changes in protein levels. An advantage of using immunohistochemistry is that it allows ascertaining the location of HSP27 in muscle biopsies. Indeed, changes in stress protein expression can occur within the muscle fibre directly responsible for muscle contraction or in other tissues such as vascular and neural structures or in the connective tissue. We found that, after resistance exercise, HSP27 tended to cluster in the fibre cytoplasm, but not at the level of the sarcolemma or the connective tissue around the fibres, giving the fibres a granular appearance. Our data also show that HSP27 relocation in response to resistance exercise occurs mainly in type II muscle fibres.
An increase in HSP27 protein content, analysed on homogenised muscle samples, has previously been shown in response to exercise involving high-force contractions (Paulsen et al. 2007; Thompson et al. 2002; Thompson et al. 2003; Thompson et al. 2001), but not following endurance exercises (Feasson et al. 2002; Morton et al. 2006). Extending these findings, and using immunohistochemistry, we found that resistance exercise, but not endurance exercises, induces the appearance of muscle fibres with granular accumulations of HSP27. The appearance of HSP27 accumulations in muscle fibres from vastus lateralis has previously been reported in response to exercise involving high-force eccentric contractions, inducing muscular damage (Paulsen et al. 2007). In response to the protocol used by Paulsen et al (2007), consisting of 300 maximal eccentric contractions, an average of 81% of the muscle fibres contained HSP27 accumulations. In our resistance exercise protocol, the number of muscle fibres containing granular HSP27 accumulations averaged 6.3% of the total number of fibres. The subjects in RE performed both concentric and eccentric contractions with the same absolute resistance and the intensity was set at a resistance level of 70% of 1 RM, which corresponds to approximately 50-60% of one maximal eccentric contraction (Westing et al. 1990). The work performed by subjects participating in our RE protocol is unlikely to induce muscular damage, as in the study by Paulsen et al (2007). Consequently we suggest that HSP27 preferentially acts to protect the myofibrillar organization during exercise causing high mechanical stress, even when muscle damage is not induced.

The utilization of the vastus lateralis varies between the two different exercise protocols used in our study. Consequently the muscle fibres are exposed to different amount and type of stress during the endurance and resistance exercises. First, according to Gollnik et al (1974), the force exerted on the pedals during cycling only amounts to about 1/3 of the maximum voluntary contraction (MVC), ranging from 15 up to 35% of MVC depending on the load and
the cadence. Thus, the muscular force exerted during one-leg cycling at 75% of VO$_{2\text{peak}}$ and 60 rpm is low compared to the force applied during resistance exercise at 70% of 1RM. Second, we have analysed glycogen content in vastus lateralis muscle in both EC and RE. There were no differences in the staining intensity of muscle fibres between biopsies taken before and after the resistance exercise (fig 5 C-D), indicating that glycogen utilization was low in the RE group. On the other hand, following the endurance cycling (EC), there was a clear glycogen depletion in muscle biopsies taken after the exercise bout, (fig 5 A-B) indicating that HSP27 accumulation is not related to exercises relying on high glycogen utilisation. These differences in vastus lateralis utilization during the two exercise protocols suggest that HSP27 is particularly sensitive to exercises including high mechanical stress on the muscle fibres rather than exercises giving rise to high metabolic stress. Interestingly; results from murine skeletal muscle (Koh and Escobedo 2004) suggest that granular accumulation of HSP25 (homolog to HSP27 in human) is restricted to lengthening contractions, as isometric contractions did not induce protein relocation. Future studies should investigate whether resistance exercise conducted with only concentric contractions would induce HSP27 relocation within human skeletal muscle fibres.

In response to RE there was a large inter-individual variability in the number of fibres showing cytoplasmic accumulations of HSP27. A similar inter-individual variability was previously reported for another member of the HSP family (HSP72) in subjects submitted to an exercise protocol consisting of treadmill running for 60 minutes at a workload corresponding to 70% of peak oxygen consumption (Walsh et al. 2001). In that study, HSP72 protein level in human vastus lateralis muscle was increased in only 2 out of 5 subjects following the exercise bout. One explanation for the inter-individual differences in the HSP27 response following the resistance exercise bout might be the existence of differences in
muscle strength between subjects. In order to assess the influence of muscle strength on HSP27 response, we analysed the relationship between HSP27 and normalized strength. Our results showed that the relocation of HSP27 in response to the resistance exercise is not correlated to the subject’s muscle strength ($r=-0.2$, $p=0.6$). An important finding in the context of training principles is the inter-individual variability in the response to similar exercise stimuli (Wilmore and Costill 1999). Our results and those of Walsh et al. (2001) suggest that the inter-individual HSP response to exercise is a possible marker for the effectiveness of the training stimulus in the process of muscle adaptation to exercise.

Our results also showed that the cytoplasm of muscle fibres displays a homogenous light staining with HSP27 antibody. The staining intensity of HSP27 was slightly higher in type II compared to type I fibres in 13 out of 15 subjects, whereas in the remaining subjects, there were no differences in the staining intensity between type I and type II muscle fibres. It has been previously shown that rat (Inaguma et al. 1993) and rabbit (Neufer and Benjamin 1996) skeletal muscle contain higher levels of HSP27 in slow oxidative compared to fast glycolytic muscles. However, the expression of HSP27 in individual muscle fibres was not assessed in these studies. Although rodent soleus might contain higher levels of HSP27 than the extensor digitorum longus (EDL), the possibility that type II fibres contain more HSP27 than type I fibres within each muscle can not be excluded. Therefore, despite inter-muscular differences in the total amount of HSP27 in rodent skeletal muscle, it is possible that within each muscle, the type II fibres contain more HSP27 than type I fibres. Alternatively, it is possible that the expression of HSP27 in mixed skeletal muscles such as human vastus lateralis is not similar to that in homogenous rodent soleus and EDL muscles. The fact that type II fibres are specialized in the production of high levels of force can explain the preferential expression of HSP27 in type II fibres in human vastus lateralis muscle as
HSP27 is suggested to play an important role in the adaptation to forceful contractions. This is strengthened by our data showing the HSP27 relocation following the resistance exercise protocol occurred only in type II muscle fibres.

In conclusion, this study demonstrates the occurrence of an early HSP27 relocation within the muscle fibres in human vastus lateralis following resistance but not endurance exercises. This enlightens the role of HSP27 as an early cellular factor important in the muscular adaptation to an exercise involving high-force contractions. It is also clear that this protein relocation occurs in some but not all subjects, highlighting the large spectrum of inter-individual variability in the response of human skeletal muscle to a similar workload. Further studies are warranted to improve our understanding of the relationship between the early cellular events to one bout of exercise and the subsequent changes in functional properties seen in response to training. This would provide a better basis for the conception of individual exercise prescriptions and the optimisation of training interventions.
Acknowledgements

This study was supported by grants from the Swedish National Centre for Research in Sports.

Conflict of interest

There are no conflicts of interest.
References


Figure legends

**Figure 1** Muscle cross-section from human vastus lateralis stained with the monoclonal antibody against HSP27. Fibres showing granular cytoplasmic accumulations of HSP27 are seen in muscle biopsies taken immediately after resistance exercise. (Scale bar = 50 µm)

**Figure 2** Immunohistochemical staining of two serial muscle cross-sections from human vastus lateralis using the antibody A4951 that strongly reacts with myosin heavy chain type I (fig A) and the HSP27 antibody (fig B). Differences in HSP27 staining intensity between type I and type II muscle fibres are seen. Fibre types I and II are denoted by I and II, respectively. (Scale bar = 500 µm)

**Figure 3** Muscle cross-sections from two subjects stained with the monoclonal antibody against HSP27. Biopsies taken before and immediately after the endurance cycling protocol (fig A,B), and before and immediately after the resistance exercise protocol (fig C,D). Cytoplasmic granular accumulations are only seen immediately after the resistance exercise protocol. Note the slightly stronger homogenous cytoplasmic staining in type II fibres in all four muscle cross-sections. (Scale bar = 100 µm)

**Figure 4** The percentage of muscle fibres containing HSP27 accumulations before and immediately after resistance exercise. HSP27 accumulations were seen in five out of nine subjects. Solid lines represent individual values and the dashed line indicates the mean response, calculated from all nine subjects. * p < 0.05, significant difference between pre and post exercise.
Figure 5. Muscle cross-sections from two subjects stained for glycogen content using the PAS-method. Biopsies are sampled before and immediately after the endurance cycling protocol (fig A,B), and before and immediately after the resistance protocol (fig C,D). Note that glycogen depletion occurs after the endurance cycling, but not after the resistance exercises (Scale bar = 100 μm)
**Tables**

**Table 1** Subject characteristics in the two different exercise protocols. Data are presented as mean ± SD. EC, endurance cycling group; RE, resistance exercise group.

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<th>Age (year)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
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<tr>
<td>EC (n=6)</td>
<td>26 ± 5</td>
<td>74 ± 9</td>
<td>172 ± 3</td>
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<td>RE (n=9)</td>
<td>24 ± 2</td>
<td>80 ± 16</td>
<td>183 ± 5</td>
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Figure 2
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