ORIGINAL ARTICLE

Effect of training and detraining on the expression of heat shock proteins in m. triceps brachii of untrained males and females

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Abstract Forty untrained persons were randomized to four different training protocols that exercised the m. triceps brachii. Group 1 and 2 performed high intensity (HI) elbow extensions and group 3 and 4 performed low intensity (LI) elbow extensions. Group 1 and 3 trained until they had accumulated a matching high volume (HV) of training, while group 2 and 4 trained until they had accumulated a matching low volume (LV) of training. Training for 5–8 weeks increased the HSP72, HSP27 and GRP75 levels in the subjects' m. triceps brachii by 111, 71 and 192%, respectively (Fig. 1a–c). There were, however, no significant differences in the heat shock protein (HSP) responses to training between the four training groups (Fig. 2a–c). The frequency of extreme responses to exercise was, however, higher after HI exercise than after LI exercise, indicating that HI exercise induces extreme HSP reactions in some subjects. When we assigned the subjects to three clusters, according to the total number of repetitions they had lifted, the subjects who had lifted the highest number of repetitions had lower PostExc HSP levels compared with subjects that lifted the lowest number of repetitions (Fig. 3a–c). Additionally, there was a negative non-linear regression (Fig. 4a–c) between the subjects PreExc levels of HSP72, HSP27 and GRP75 and the percentage change in their respective protein concentration after training ($r = -0.75$, -0.89 and -0.88, all $P < 0.0001$). Thus, the PreExc level of HSPs seems to be an important "regulator" of HSP expression following the training.

Keywords HSP72 · HSP27 · GRP75 · Intensity · Volume · Duration · Untrained muscle

Introduction

Heat shock proteins (HSPs) or "stress proteins" are cytosolic proteins that are highly inducible by several factors, including exercise. Acute exercise of various kinds generates a relatively rapid and transient increase in HSP expression in human tissues (Febbraio and Koukoulas 2000; Khassaf et al. 2001; Puntschardt et al. 1996; Ryan et al. 1991; Shastry et al. 2002; Thompson et al. 2001, 2003). However, relatively few studies have focused on the HSP response to long-term exercise in human skeletal muscle and even less so, on how different intensity and different volume of the exercise affect the expression of various HSPs. Results from animal studies on HSP expression after different amounts of training are equivocal. Morán et al. (2004) observed no significant response in the HSP72 expression in rat muscle after 12 weeks of endurance running, while there was a threefold increase after 24 weeks of training. Ecochard et al. (2000) showed that 2 weeks of exercise was necessary to elevate rat muscle HSP72 content, but there was no further change in HSP72

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with additional training. In man, the situation is very much the same. Liu et al. (1999) investigated the HSP70 response in trained humans exposed to 4 weeks of rowing exercise by taking serial biopsies after each week of training. The expression of HSP70 increased in relation with increasing amounts of exercise, and they suggested that the HSP70 response was related to the total amount of exercise. In a later study where the total training volumes were matched between two groups of rowers, while the training intensity was different, Liu et al. (2000) showed that HSP70 increased with training and that the increase in HSP70 was affected by the training intensity. Thus, the question of how exercise volume and intensity affects the HSP response seems unresolved and warrants further investigations. We address this topic in the present study by using a design that allows for comparison of high intensity (HI) and low intensity (LI) training each with two matching volumes (high and low) of training.

This may provide new insight about the impact of training intensity and training volume on the HSP response in human skeletal muscle. Previous research has indicated that the variability in the response of HSPs between subjects is substantial with some subjects showing very large responses while others show only minor responses to exercise (Boshoff et al. 2000; Khassaf et al. 2001). It has been suggested that this partly may be due to a proportionately smaller response in those subjects with relatively high baseline levels of HSPs (Khassaf et al. 2001). This interesting topic has received little attention, and we will look more closely into this matter in the present study. Only few studies have investigated the effects of reduced contractile activity (detraining) on the expression of different HSPs, and the results from animal models of detraining are equivocal (Naito et al. 2001; Oishi et al. 2001; Desplanches et al. 2004). In man, there is scarce information regarding the effect of detraining on HSP expression in skeletal muscle. Liu et al. (2004) observed a significant decrease in HSP expression after 1 week of recovery following 3 weeks of HI exercise. Another 3 weeks of endurance training immediately following the recovery period did not elevate the HSP content in the vastus muscle any further, and the HSP level remained unchanged after another week of detraining subsequent to the endurance training. In the present study, the training period is longer than in the study of Liu et al. (2004), and we have examined the HSP response after 8 weeks of detraining.

The purpose of the present study is fourfold. Firstly, to investigate the effect of long-term exercise on HSP expression in a previously untrained human skeletal muscle. Secondly, to investigate the impact of differing training intensities and training volumes on the HSP expression in the previously untrained muscle. Thirdly, to investigate whether there are individual differences in the HSP response to exercise that may be attributed to the baseline level of the different HSPs. Finally, to investigate the effect of detraining on exercise-induced HSP expression subsequent to long-term exercise.

Materials and methods

Subjects

Seventy-seven healthy males and females volunteered to participate in this study. Prior to inclusion all persons completed two questionnaires, one about self-reported health and another about habitual physical activity. Based on the results from a 1RM test of their elbow extensors and the questionnaires, we excluded 37 of the 77 volunteers from the study to make the experimental group homogenous with respect to previous training history and elbow extensor strength. We excluded persons with a history of regular exercise of the elbow extensors and those with the highest and the lowest 1RM. The remaining 40 subjects were subsequently freely randomized to the different training regimes according to a computer-generated protocol. There were eight dropouts from the study (three men and five women). Six left the study prior to the post-exercise biopsy, and two dropped out just after the pre-exercise biopsy. The remaining subjects were generally fit and healthy and were not using any medication for chronic diseases. They had not been engaged in any type of regular exercise of the elbow extensors during the last 6 months prior to this study. During the study, the subjects were instructed not to engage in physical activities that exercised their triceps muscle other than their prescribed training programme. The subjects' adherence to this regimen was controlled by their self-reported training diary. The mean (SE) age, height and weight of the 32 persons (12 males and 20 females) who completed this study were as follows: age 22.0 (0.6) years, height 172.8 (1.3) cm and weight 64.6 (1.2) kg. Written informed consent was obtained from all subjects, and the study was approved by the Regional Committee for Medical Research Ethics.

Study design and training protocol

The study was a randomized design with two independent variables, which are the *intensity* of training and the *volume* of training (Table 1). For each independent variable, there were two levels: *high* or *low*. Group 1

Table 1 Training programme and study design

Groups	Training mode	Intensity, % 1RM	Volume, intended tons	Sessions (week^{-1})	Average total number of sessions
1. HI–HV	Intermittent	60 (HI)	200 (HV)	3	23
2. HI–LV	Intermittent	60 (HI)	100 (LV)	3	18
3. LI–HV	Continuous	30 (LI)	200 (HV)	3	23
4. LI–LV	Continuous	30 (LI)	100 (LV)	3	15

Group 1, n = 1 male and 5 females; group 2, n = 2 males and 3 females; group 3, n = 6 males and 7 females; group 4, n = 3 males and 5 females

HI high intensity, *LI* low intensity, *HV* high volume, *LV* low volume

and 2 both trained at HI. Group 1 accumulated a high volume (HV) of training while group 2 accumulated a low volume (LV) of training. Group 3 and 4 both trained at LI. Group 3 accumulated the same volume of training as group 1 (HV), while group 4 accumulated the same volume as group 2 (LV). The subjects in all groups performed their training (triceps extensions) with both arms simultaneously in a commercial training apparatus (Triceps Extension Machine, Cybex International Inc., USA).

Intensity of training

The HI training consisted of intermittent elbow extensions at 60% of the subjects' 1RM. One *series* of exercise consisted of 20 repetitions during 30 s, paced by a metronome. Five series constituted one *set* (i.e. 100 repetitions) and there was a 30-s break between each series. The subjects completed four sets per session (4 × 100 repetitions), with a 5 min rest period between each set.

The LI training consisted of 40 elbow extensions per minute (which is the same pace as for the HI training) continuously for 20 min at 30% of the subjects' 1RM. A metronome paced the training. Thus, the HI subjects lifted 400 repetitions per session, while the LI subjects lifted 800 repetitions per session. The maximal strength of the elbow extensors (1RM) was tested every week, and the training load was adjusted progressively to keep the intensity at the designated percentage of the subjects' 1RM.

Volume of training

The total volume of training is calculated as the total weight lifted per session times the total number of sessions. Since the 1RM varied somewhat between the subjects, we used different total number of sessions to achieve equal training volumes within the different groups. Thus, to achieve the same absolute volume of

training, the subjects with the lower 1RM had to perform more sessions than the stronger subjects. The LV training was pre-set to a total of ∼100 ton, while the HV training was pre-set to a total of ∼200 ton. The subjects trained three sessions per week on alternating days, until they had completed their designated volume of training. The subjects exercised for an average of 5 weeks for the LV group and 8 weeks for the HV group.

Detraining

After completion of the training period the subjects went through a period of detraining that lasted 8 weeks. During this period, the subjects were instructed to return to their normal daily lifestyle and resume their activity level prior to this study. The subjects were also instructed to refrain from regular training of the elbow extensors during the detraining period.

Physiological monitoring of training

To characterize the physiological demands of the differing training regimes, we measured oxygen uptake, lactate, heart rate and perceived exertion during ordinary training for all subjects. All measurements were taken 3 weeks after commencing the training programme. Oxygen uptake was measured by the Douglas bag method, heart rate was measured by a Polar SportsTester ME 3000 (Polar Electro, Finland), capillary blood lactate was analysed on an YSI 23 (Yellow Spring Instruments, USA) lactate analyser and ratings of perceived exertion (RPE) was assigned according to the Borg category ratio scale (Borg 1982).

Choice of muscle

Most training studies have chosen to study the exercise response in locomotor muscles like the m. vastus

lateralis. This makes it, however, difficult to separate the effect of specific training from the added effect of daily locomotor and postural activity that these muscles are exposed to. In the present study we chose to exercise a non-locomotor muscle (m. triceps brachii) and this makes it possible to minimize the influence of other contractile activities than the prescribed training on the adaptive processes in the triceps muscle.

Biopsy protocol

A needle biopsy (Bergström 1962) was taken from the lateral head of m. triceps brachii of the non-dominant arm prior to training (PreExc), after termination of the training period (PostExc) and then 8 weeks after the PostExc biopsy (detraining). The biopsy samples were frozen in isopenthane cooled in liquid nitrogen and stored at $-70°C$ until analysis. Care was taken to obtain the pre-exercise, post-exercise and detraining biopsies from the same area and depth of the muscle belly.

Biochemical analysis

Pieces of frozen biopsies were transferred to Eppendorf tubes kept on ice. Ice-cold homogenization buffer (Guirreiro et al. 1989) was added to the Eppendorf tubes, and the biopsies were then homogenized with short bursts of a motorized pestle. The homogenate was centrifuged at $4°C$ at $10,000g$ for 20 min. The supernatant was transferred to Eppendorf tubes kept on ice and stored at $-70°C$ until analysis. The total protein concentration of the samples was determined using the bicinchoninic acid (BCA) protein assay.

Muscle homogenates were diluted with sample buffer (Laemmli 1970) and heated for 4 min at $100°C$ in a water bath. A discontinuous gel system with a 4% stacking gel and a 10% separating gel was used for separation of proteins (BioRad Mini-Protean 3). Electrophoresis was run at 200 V.

Separated proteins were transferred from gels to 0.2 μm PVDF membranes (BioRad Mini Trans Blot system) according to Towbin et al. (1979). Protein transfer was done at 100 V. The membranes were blocked with 5% skimmed milk powder in 0.1% TBS-Tween on a shaker over night at $4°C$. After blocking, membranes were incubated with the following antibodies: Anti-HSP72 (StressGen SPA-810), anti-GRP75 (StressGen SPA-825) or anti-HSP27 (StressGen SPA-800). After incubation with primary antibody, membranes were rinsed in TBS-Tween before incubation with a horseradish peroxidase conjugated secondary antibody (StressGen SAB-100) and then subsequently

rinsed in TBS-Tween before incubation with an ECL + kit (Amersham RPN 2132) for detection of protein bands.

Quantification

Developed X-ray films (ECL Hyperfilm, Amersham) were scanned, and digitized bands were subsequently analysed and quantified by image analysis software (TotalLab, Nonlinear Dynamics, UK). Before quantification, each digitized scan was calibrated against an optical density step-wedge. Values for HSP72 and HSP27 were normalized against known amounts of recombinant human HSP72 (StressGen SPP-755) and HSP27 protein (StressGen SPP-715) run on the same gel as the experimental samples. Recombinant human GRP75 protein is not commercially available, and thus it is not possible to normalize the blots against known amounts of GRP75. Therefore, HeLa heat shocked cells lysate (StressGen LYC-HL101F) was used as a positive control for GRP75, and values for GRP75 are reported as mean *integrated optical density* (mean IOD) units. IOD is calculated as the sum of pixel grey level values within a defined area divided by the number of pixels within the same area.

Statistical analyses and data analysis

Normal distribution plots (Q–Q plots) of the changes in the net concentrations (PostExc values minus PreExc values) of the HSPs versus the expected normal distribution showed that the data with the exception of two or three outliers were fairly normally distributed. Separate tests showed, however, that these outliers had a considerable effect on the outcome of statistical tests if standard analysis of variance (ANOVA) and t tests were carried out. To minimize the influence of outliers on the statistical analysis and because the variances between the variables were uneven, we have chosen robust methods (non-parametric) for the analysis of our data. Main differences between PreExc, PostExc and detraining values were initially tested with Friedman's repeated measures test. When significant differences were detected, we used the Wilcoxon paired samples test for further post-testing. The Kruskas–Wallis test was used to test for differences between the different training groups and for possible effects of the number of repetitions on the HSP concentration. When significant differences were detected, we used the Mann–Whitney U test for further post-testing. Correlations were tested with the Spearman's rho test. Regression analysis was performed to define the relationship between basal HSP levels (PreExc values) and

Table 2 Physiological characteristics of HI and LI and exercise

Training intensity	VO$_2$ (L/min)	Heart rate (beats/min)	Lactate (mmol/L)	RPE, scale 1–10
HI (*n* = 11)	1.15 (0.1)	129.5 (1.5)	3.6 (0.2)	5.7 (0.4)
LI (*n* = 21)	0.62 (0.04) **	98.5 (4.4) **	1.9 (0.2) **	3.1 (0.2)**

VO$_2$ values are sampled midway in the respective training sessions. Heart rate and lactate levels are average values after registrations every 5 min for group 3 and 4. For group 1 and 2 heart rate and lactate values are average values of registrations from the last series in set 2 and the last series in set 4. RPE are registrations immediately after the training session. Values are means (SE)

HI training = group 1 and 2, LI training = group 3 and 4

**$P < 0.01$ compared with HI training

the exercise-induced changes in the level of HSPs. Subjects are classified as "responders" when the individual net protein concentration (PostExc minus PreExc values) increased following training. They were classified as "non-responders" if the net protein concentration was reduced following training.

Statistical calculations were done with SPSS statistical software (SPSS Inc., USA). Statistical significance was set at $P < 0.05$. Data are presented as means (SE).

Results

Physiological responses and completed training

There were no statistical differences between the different training groups regarding physical characteristics such as age, height, weight and PreExc 1RM. On the other hand, values for oxygen uptake, heart rate, lactate and RPE during LI training averaged 54, 76, 53 and 54% of HI values, respectively ($P < 0.01$ for all). Thus, the metabolic and physiological stress during HI training was quite different from the LI training (Table 2). There were no gender differences in the responses of these variables. The LV subjects lifted on average 108 (2,145) ton, while the HV subjects on average lifted 180 (2,250) ton. Thus, the LV subjects achieved on average 60% of the total lifting volume of the HV subjects ($P < 0.0001$).

Stress protein responses

PreExc levels of the different HSPs were not different between males and females or between the different training groups. The main effect of long-term training on the expression of HSPs is shown in Fig. 1a–c. Long-term training increased the mean levels of HSP72 by 111% ($P < 0.005$) and after 8 weeks of detraining, HSP72 levels were declining, but were still elevated compared with PreExc levels ($P < 0.05$).

Mean levels of HSP27 increased by 71% ($P < 0.002$) after training. After detraining the HSP27 levels were reduced compared to PostExc values ($P < 0.01$), and the detraining levels were similar to PreExc levels.

Mean levels of GRP75 increased by 192% ($P < 0.0001$) after training, and after detraining the GRP75 levels were reduced compared to PostExc levels ($P < 0.04$), and were similar to PreExc levels ($P = 0.06$).

Effect of training intensity and volume

We observed no differences in net HSP72, HSP27 or GRP75 levels between the four different training regimes (Fig. 2a–c). Despite this, inspection of the figures may suggest that HI training is important in elevating the levels of these proteins. A chi-square test showed that the observed frequency of outliers (extreme values) after HI training was higher than the expected frequency ($P < 0.001$). We also calculated the net changes in HSP levels (detraining values minus PostExc values) following 8 weeks of detraining. There were no significant differences in the detraining response between the different training groups for any of the proteins.

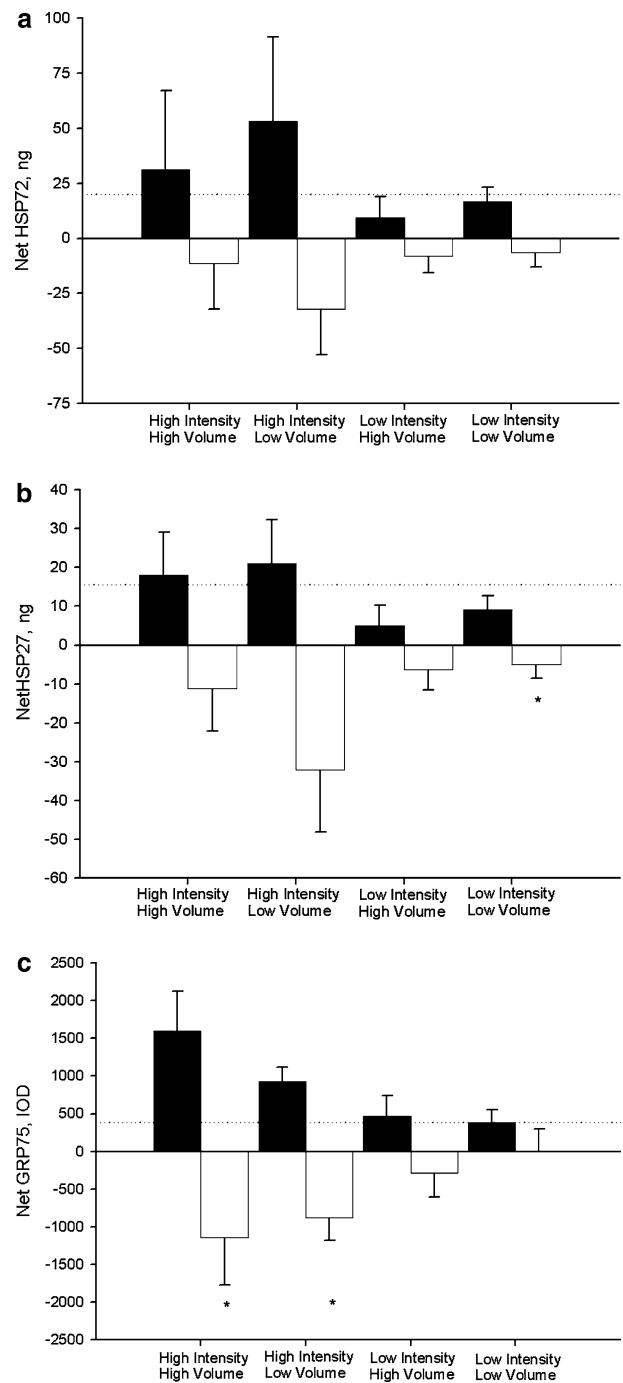### Relationship between number of repetitions and PostExc HSP levels

We divided the subjects into three bins according to the total number of repetitions they had carried out: subjects that had lifted 5,000–11,000 repetitions ($n = 12$), 12,000–17,000 repetitions ($n = 10$) and 18,000–22,000 repetitions ($n = 10$), respectively. For all three HSPs, there was an optimum training duration or number of repetitions that resulted in the largest increase in protein concentration. For all HSPs, the higher values were obtained in individuals that lifted 5,000–11,000 repetitions (Fig. 3a–c). More repetitions (training for a longer period) led to lower concentrations of HSPs. There were no effects of gender on these variables for any of the HSPs.
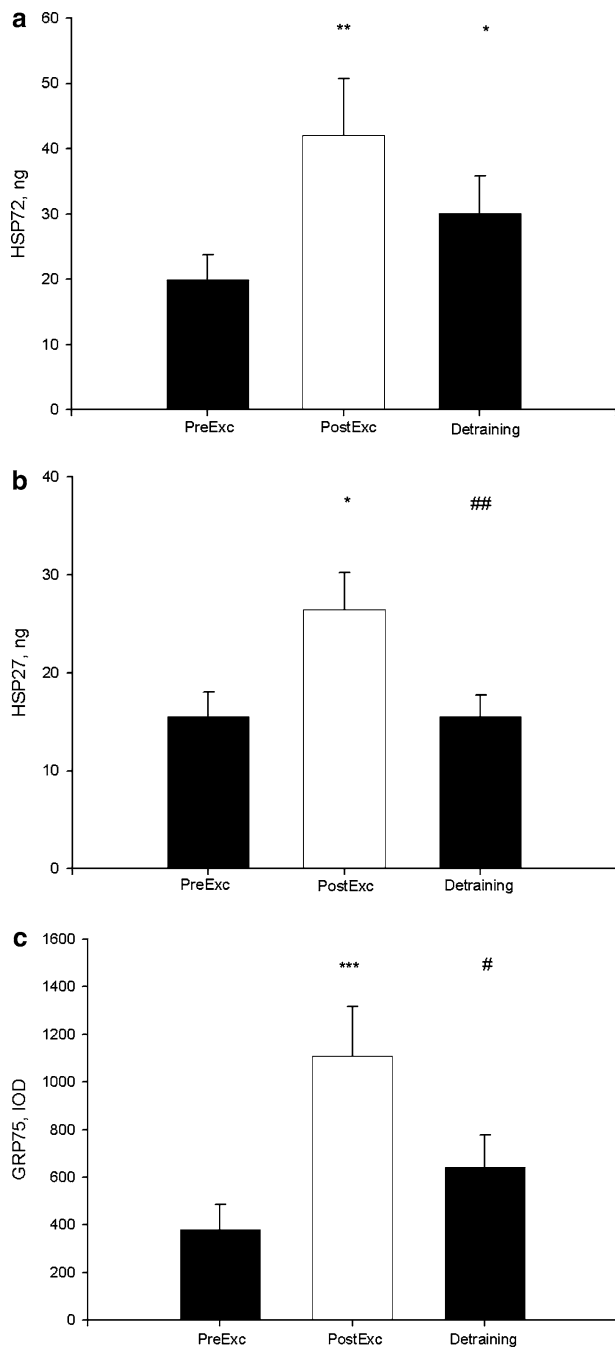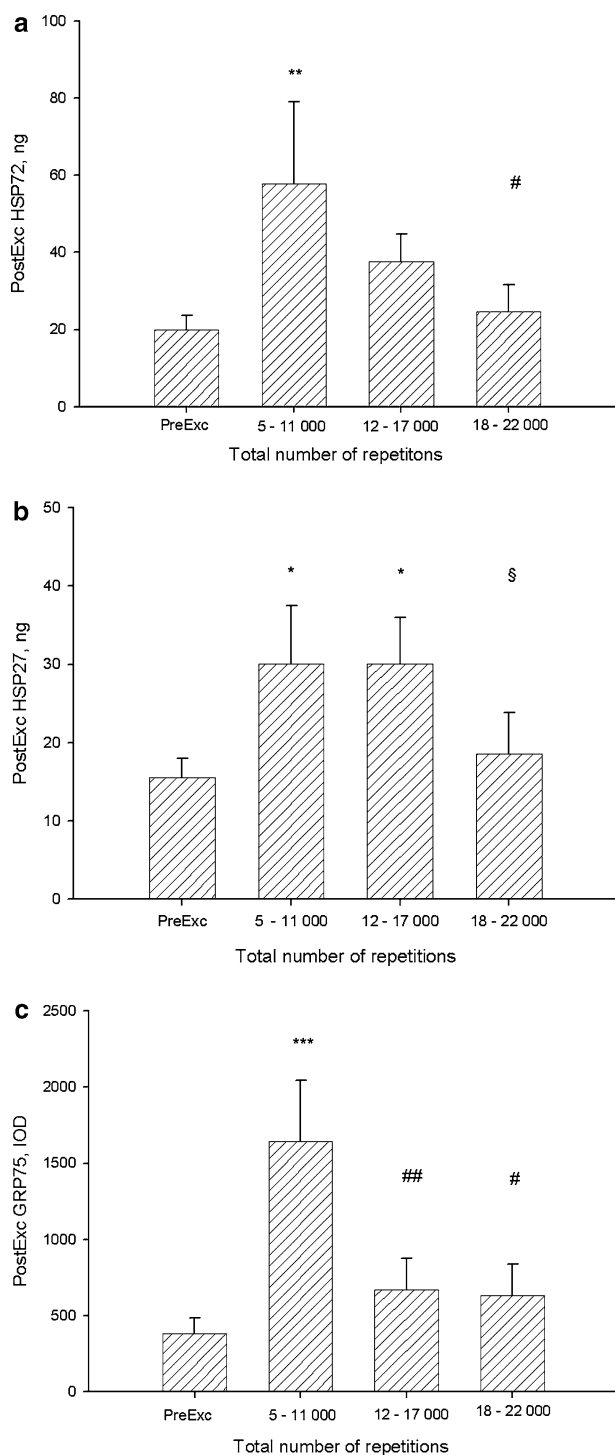
Fig. 1 a–c HSP72, HSP27 and GRP75 response to long-term training. *Ng* nanogram, *IOD* integrated optical density. $N = 32$ for PreExc, PostExc and detraining. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$ compared to PreExc values. $^{\#}P < 0.04$, $^{\#\#}P < 0.01$ compared to PostExc values. Values are means (SE)

Fig. 2 a–c Net HSP72, HSP27 and GRP75 protein concentration in response to long-term training and detraining. *Black columns* = PostExc values minus PreExc values (net accumulation), *white columns* = detraining values minus PostExc values (net reduction). *Ng* nanogram, *IOD* integrated optical density. HI–HV group $n = 1$ male and 5 females; HI–LV group, $n = 2$ males and 3 females; LI–HV group, $n = 6$ males and 7 females; LI–LV group, $n = 3$ males and 5 females. *Dotted line* = mean PreExc values for all four training groups collectively. $*P < 0.05$ = net accumulation compared with net reduction. Values are means (SE)

Relationship between basal levels (PreExc) of HSPs and change in protein concentration

Our principal analyses uncovered a large scatter in the individual HSP responses to exercise. Regression analy-

Fig. 3 a–c HSP72, HSP27 and GRP75 response in relation to the total number of repetitions lifted. The subjects are divided into bins according to the total number of repetitions they lifted during the study. PreExc = 12 males and 20 females, 5,000–11,000 repetitions = 5 males and 7 females, 12,000–17,000 repetitions = 4 males and 6 females, 18,000–22,000 repetitions = 3 males and 7 females. *$P < 0.02$, **$P < 0.004$, ***$P < 0.001$ compared with PreExc values. #$P < 0.04$, ##$P < 0.01$ compared with 5,000–11,000 repetitions. §$P = 0.06$ compared with 12,000–17,000 repetitions. *Ng* nanogram, *IOD* integrated optical density. Values are means (SE)

sis of individual responses showed that the increase in HSPs during the training period was negatively related to the PreExc level of the respective HSP. For HSP72 the correlation was −0.75, for HSP27 the correlation coefficient was −0.89 and for GRP75 the coefficient was −0.88, all $P < 0.0001$ (Fig. 4a–c, outliers excluded). There was no effect of gender on the regression analysis.

Discussion

Although studies in man are few, there seems to be the consensus that HSP70 concentrations increase after some weeks of training (Liu et al. 1999, 2000, 2004; Vogt et al. 2001). The present report of a 111% increase in HSP72 concentration after 5–8 weeks of training is in line with this. Previous studies in animals have also reported increases in HSP27 and GRP75 after long-term training or chronic electrical stimulation, but the present study is the first to report increases in the GRP75 and HSP27 levels in skeletal muscle of man after prolonged training.

The results from the present study appear to be in contrast with our previous study (Gjøvaag et al. 2006). In our earlier study, very well trained males performed HI strength training for 12 weeks, and we observed a decrease in HSP72 concentration, while the GRP75 levels remained unchanged following training. Although the training regimens in these two studies are clearly different, we believe that the main reason for the differences in outcome is different training status of the subjects. In the present study, we recruited subjects with objective criteria for being untrained in the exercising muscle, while the subjects in our previous study were athletes with a record of extensive training of the exercising muscle. Studies on other well-trained subjects (rowers) have shown that the net accumulation of HSP72 (post-values minus pre-values) decreases with increasing length of training (Liu et al. 1999). This is also in agreement with a study by Belter et al. (2004) who showed that on the individual level, the amount of wheel running during 1 week had a significant negative effect on HSP72 expression in mouse m. triceps surae. One interpretation of these results is that as muscles become increasingly better trained (e.g. like athletes) there is an attenuation of the HSP response due to cellular regulatory mechanisms. Thus, we argue that when untrained subjects start to exercise, there will be an initial increase in their HSP concentration (Fig. 1a–c) followed by a later decline in their HSP response as the training progresses.

This is supported by our findings that those of our subjects who lifted more repetitions ("the better trained") had lower PostExc HSP levels compared with
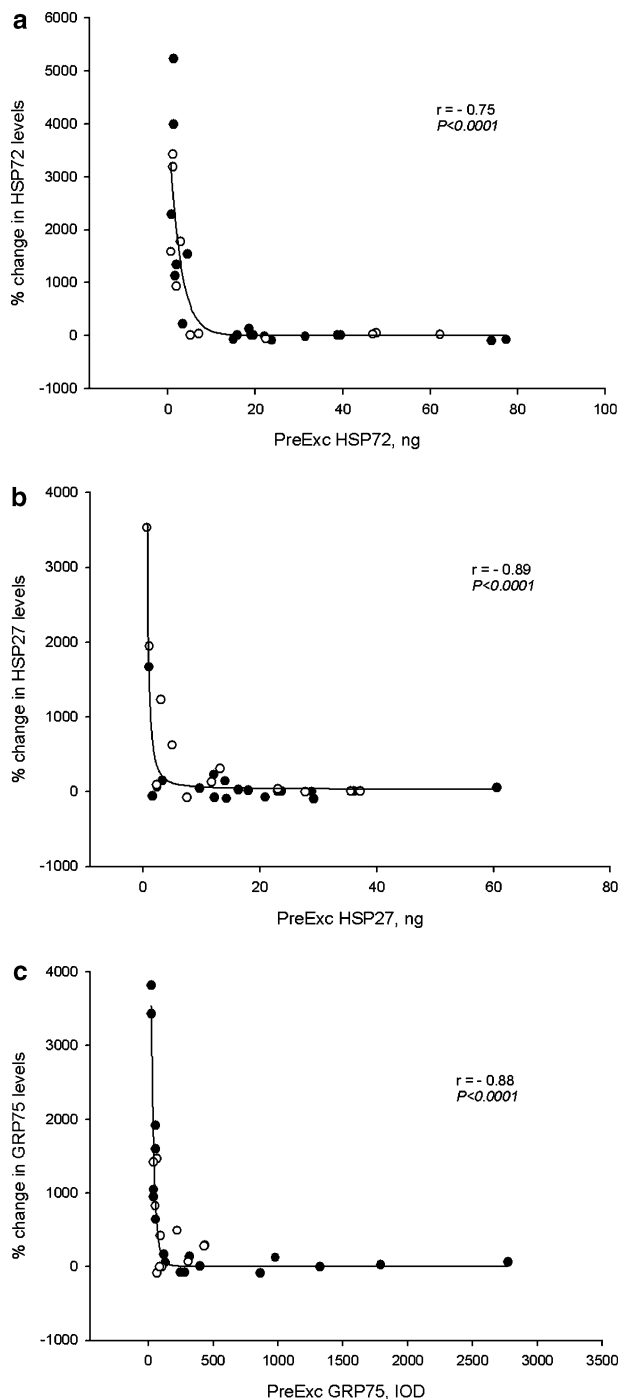
Fig. 4 a–c Regression analysis defining the relationship between individual PreExc levels of the heat shock proteins and their percentage change following training. *Ng* nanogram, *IOD* integrated optical density. Outliers are excluded. *N* = 30 for HSP72 and HSP27 and *n* = 29 for GRP75. *Closed symbols* = females, *open symbols* = males

the subjects who lifted fewer repetitions (Fig. 3a–c). Thus, very well trained muscles may have a different HSP response than untrained muscles. This is also in line with reports of lower basal levels of HSP70 and

HSP27 positive leukocytes in trained runners compared with untrained individuals (Fehrenbach et al. 2000a, b).

The effect of training intensity

There is an ongoing discussion whether HSPs respond more to HI exercise than to LI exercise (Liu et al. 2004) and one of the main objectives in the present study was to investigate the impact of differing training intensities and training volumes on the HSP expression in the previously untrained muscle. We found, however, no significant difference for HSP72 and HSP27 regarding the intensity or the volume of exercise. Despite non-significant findings, we find it worthwhile to comment on Fig. 2a–c. For all HSPs the pattern seems to be that LI exercise, in contrast to HI exercise, is unable to stimulate to increased netHSP (PostExc−PreExc values) concentration. As can be seen in Table 2, the oxygen consumption, heart rate and lactate levels during LI training are fairly moderate and the physiological stress generated by LI exercise may thus be insufficient in stimulating to increased HSP production.

Evaluation of our data also showed that the variability of the individual responses was large, especially among the subjects that performed HI exercise. Hence, the lack of significant differences between HI and LI training may be due to a number of extreme values among the HI subjects. Based on *Q–Q* plots we have earlier classified seven changes during the training as outliers. Further analysis identified that six of the seven outliers (two outliers from HSP72, two from HSP27 and two from GRP75) were from subjects exposed to HI training. No subject showed more than one such extreme value. This means that six of 11 subjects that were exposed to HI training showed one extreme HSP concentration after the training period. This is in contrast to the response after LI training where only one of 21 subjects showed an outlier (from GRP75). A chi-square test showed that the observed frequencies of outliers after HI training and LI training were different from the expected frequencies ($P < 0.001$), and it is thus highly probable that HI training per se induces extreme reactions in some subjects. Also the studies of Vogt et al. (2001) and Liu et al. (2004) support the view that exercise intensity is important in stimulating the HSP response. Both of these studies showed that HSP72 levels increased by HI training, but not by LI training. Several authors have suggested that the increased HSP72 response following HI training may be an effect of high lactate concentrations during exercise. Both Vogt et al. (2001) and Pösö et al. (2002)

found a positive correlation for HSP72 mRNA levels and lactate levels during long-term and acute exercise, respectively. In the present study we did also find a positive correlation between PostExc HSP72 and HSP27 concentration and the blood lactate concentration during training ($r = 0.45$ and 0.35, $P < 0.01$ and $P < 0.03$, respectively), but no such correlation was found for GRP75. A causal relationship between high lactate levels and the induction of HSPs has however not yet been established, consequently one need to exercise caution when considering the influence of lactate per se on HSP expression. However, it should be noted that we measured the blood lactate concentration and not that in muscle. The blood lactate concentration is only a crude measure of that in the muscles (Medbø et al. 2001). It may also be that there are other factors besides the lactate concentration which may influence the expression of HSPs, and we will address this question briefly in relation to GRP75 (mitochondrial HSP70) and cytochrome *c* oxidase (COX). GRP75 (mtHSP70) is involved in the translocation of precursor proteins into the mitochondria (Gambill et al. 1993), whereas COX is the terminal member of the electron transport chain. A well-known adaptation of endurance training is an increase in mitochondrial content and oxidative capacity in the active muscle fibres, and in this connection, one relevant question is whether there are coordinate changes in the expression of GRP75/mtHSP70 and COX following exercise. We find, however, no correlations between GRP75/mtHSP70 and COX in our samples (data not shown), which is in accordance with several other publications (Ornatsky et al. 1995; Mattson et al. 2000; Samelman et al. 2000). Rassow et al. (1994) argues that during import of proteins into the mitochondria, as much as 85% of the GRP75/mtHSP70 remains unbound and soluble in the mitochondrial matrix. Thus, a direct correlation between mitochondrial content and GRP75/mtHSP70 expression is not required to assure adequate biogenesis of mitochondrial proteins (Mitchell et al. 2002).

The effect of training volume

The influence of training with different duration or volume of training on the HSP response is little investigated, but several authors have indicated that there may be an effect of training duration on HSP expression. In the present study, the LV subjects accumulated an average training volume of 108 ton, while the HV subjects achieved an average volume of 179 ton. Despite this large difference in training volume, there were no significant differences in protein concentration between LV and HV training for any of the investigated HSPs.

Our findings are seemingly in contrast to the study by Belter et al. (2004) who showed that on the individual level, the amount of wheel running for 1 week had a significant negative effect on HSP72 expression in mice m. triceps surae. To investigate this relationship further, we plotted our HSP data on a more continuous scale (i.e. number of elbow extensions) instead of the dichotomous (high–low) volume scale. The subjects in the present study varied in strength, but all subjects within the same group carried out similar amounts of training as evaluated by the integrated weight lifted (total "tons" lifted). Since each subject within one group carried out the same number of lifts per training session, the strongest subject completed the training programme in the shortest time. Consequently, the total number of lifts or repetitions carried out is also a measure of the training duration. These analyses showed clearly that there is an initial increase in the PostExc HSP72 levels when the numbers of repetitions increased from zero (PreExc) to between 5,000 and 11,000 repetitions. Compared to PreExc levels, HSP72, HSP27 and GRP75 levels for the subjects that lifted 5,000–11,000 repetitions increased by 191, 94 and 334%, respectively. Subjects who lifted more than 18,000 repetitions showed however, an attenuated PostExc expression of HSP72. We observed the same general tendency for HSP27 and GRP75, which is an initial rise in protein concentration, and then a substantial decline in protein levels with increasing number of repetitions (Fig. 3a–c). The subjects that lifted more than 18,000 repetitions all performed LI (HV) training, whereas the subjects that lifted less than 11,000 repetitions, mainly performed HI training. Thus, it can be argued that the rise and fall of HSP proteins in relation to the number of repetitions is an effect of different training intensities. In fact, Liu et al. (2004) argue that 3 weeks of LI rowing in contrast to HI training do not induce HSP70 in well-trained rowers. However, the subjects in our study were all untrained and there may be differences in the HSP response between well-trained and untrained subjects (e.g. Fehrenbach et al. 2000a, b). By inspection of individual data, we find that 11 of 11 subjects that trained with LI, but less than 18,000 repetitions, showed increases in net HSP72 values. In contrast, five of 10 subjects that trained with LI, but lifted more than 18,000 repetitions showed declines in net HSP72 values. Chi-square statistics showed that the observed frequency of subjects with a decline in net HSP72 values was higher among the LI subjects that lifted more than 18,000 repetitions than the expected frequency ($P < 0.007$).

Based on this, we suggest that the present findings imply that the heat shock response not only depends on the exercise intensity, but also on the exercise duration. It seems that after an initial increase in PostExc expression, the PostExc heat shock expression gradually declines with increasing number of repetitions. This interpretation is consistent with the previously mentioned observations of Belter et al. (2004) who showed that the mice with the longest training period had the lowest expression of HSP72 in the exercised muscles. In a previous study (Gjøvaag et al. 2006), we observed that in very well trained subjects, the HSP72 levels in the m. biceps brachii decreased after 12 weeks of intensive training. Thus, it may be a general phenomenon that as muscles get better and better trained, there is a reduced accumulation of HSPs in skeletal muscle. Based on these combined results, we hypothesize that all the subjects that participated in this study experienced an early and large increase in the HSP expression. We thus suggest that if those who lifted 5,000–11,000 repetitions had continued their training, their expression levels would probably have declined to levels comparable to those found in the subjects who lifted more than 12,000 repetitions. Since we did not take serial biopsies during the training period, we are unable to tell whether this rise and fall in HSP expression is present on the individual level as well. However, an attenuated HSP response in relation to the training duration seems to be in accordance with general mechanisms for regulation of HSP gene expression.

Why is the level of HSPs attenuated with increasing duration of exercise?

Apparently, the transcription of the HSP70 gene is attenuated after its initial activation despite the continuous presence of heat stress over several hours (Mosser et al. 1988). Expression of HSP70 genes is regulated by heat shock elements (HSE), which are located in the promoter region of the HSP70 genes (Amin et al. 1988). Following stress, heat shock factors (HSF) are activated and bind to the HSE, and this initiates transcription of the gene (Pelham 1982). A self-limiting mechanism for HSP synthesis has been proposed by which cells "measure" the level of stress and regulate the HSP synthesis according to the intensity of the stress (DiDomenico et al. 1982). It is not known whether all HSPs are subjected to self-regulation, but strong evidence supports that at least HSP70 is directly involved in regulating its own synthesis. DiDomenico et al. (1982) have shown that the synthesis of HSP70 is repressed precisely when a specific quantity of this protein has accumulated. A self-limiting mechanism may

attenuate the HSP70 transcription by release of bound HSF from the HSE of the HSP70 gene promoter (Abravaya et al. 1991). This is consistent with observations from, e.g. Mosser et al. (1993) who showed that over-expression of HSP70 in transfected human cells is associated with an accelerated loss of HSF binding to DNA and a subsequent attenuation of HSP70 induction. In addition, a second heat shock following recovery from a previous heat shock induces a much lower level of HSP70 transcription and a more rapid attenuation of the response (Mizzen and Welch 1988). Based on these observations it seems plausible that cells that are regularly exposed to stress (e.g. long-term exercise) express lower basal levels of HSPs than cells that are less frequently stressed.

Relationship between basal levels (PreExc) and change in protein concentration

To further look into possible factors, which may influence the HSP expression in skeletal muscle on the *individual* level, we analysed the relationship between the basal levels of the HSPs and their respective change in protein concentration (Fig. 4a–c). Overall, we found that the increase in HSPs during the training period was negatively related to the PreExc level of the respective HSPs. Individuals with very low PreExc values generally had large increases in the protein concentration following training, while individuals with high PreExc levels had lower protein levels following training. When we tested for a non-linear relationship between the percent change in protein levels and PreExc levels, there was a good fit for all the HSPs.

Interestingly, Boshoff et al. (2000) observed the same phenomenon in peripheral blood monocytes after an in vitro heat shock. Boshoff et al. (2000) found that increasing levels of basal HSP70/HSC70 synthesis were accompanied by an exponential decrease in the percentage change of HSP70/HSC70 concentration. It is possible that HSPs in white blood cells play a different role than they do in skeletal muscle (Campisi and Fleshner 2003), but the observations of Boshoff et al. (2000) are interesting in light of evidence of previous mentioned evidence of a possible regulatory role on protein expression for HSP70. To analyse whether gender had any influence on the outcome of the training response, we divided the subjects into "responders" and "non-responders". Chi-square analysis showed that the observed frequency of males and females among responders and non-responders was similar to the expected frequency. Thus, there were no significant effects of gender on the regression analyses.

The effect of detraining

There are few studies on how detraining affects HSP expression in man, and the results regarding short-term detraining are contradictory. Liu et al. (2004) showed that 1 week of detraining subsequent to 3 weeks of exercise was sufficient to reduce the HSP72 expression in human muscle. Khassaf et al. (2001) on the other hand, observed that mean muscle HSP70 level after 6 days of recovery (following a single bout of exercise) was significantly elevated compared with values obtained immediately post-exercise. Thus, at present it is not possible to conclude how short-term recovery (detraining) affects HSP expression in man. In the present study, 8 weeks of detraining reduced both the HSP27 and GRP75 levels significantly compared to PostExc values. HSP72 detraining levels were also lower than PostExc values, but this difference was not statistically significant. Results from other, more long-term detraining studies are lacking. Desplanches et al. (2004) showed however, that 2 weeks of hindlimb unloading after 6 weeks of endurance training completely reversed the training-induced accumulation of HSP72 is rat m. soleus and m. plantaris. Thus, long-term recovery seems able to reduce the training-induced HSP-accumulation towards PreExc levels in both man and rodents, but studies in humans are few and it is difficult to conclude in this matter.

The biological significance of our findings

In response to acute stress (e.g. exercise) muscle cells rapidly increase their production of different HSPs. An increased accumulation of HSPs is thought to aid in restoring cellular homeostasis, promoting cellular remodelling and providing the cells with protection against future stress (McArdle and Jackson 2002). The general contention is that when muscle cells are exposed to a "priming stress" (e.g. exercise) that activates the HSP response, this in turn protects the cells against further damage (i.e. increased stress tolerance). In general, HSPs may contribute to cellular protection by preventing protein aggregation, by assisting in refolding of damaged proteins and by chaperoning nascent polypeptides along the ribosomes (Powers et al. 2001). In line with this, we show that previously untrained muscles respond to long-term training by a large increase in the expression of HSP72, HSP27 and GRP75. However, the concept of HSPs as "molecular chaperones" is not without controversy, and it is interesting that several lines of evidence suggest that HSP overexpression may not be beneficial under all circumstances. In accordance with this, it is noteworthy that

increasing basal levels of HSPs are accompanied by an exponential decrease in the percentage induction of HSP synthesis both in immune cells (Boshoff et al. 2000), and in skeletal muscle (present study). Since the individual HSP response to exercise seems related to the basal concentration of HSPs, this indicates that there may be limits to the HSP expression in stressed cells. In light of this, Theodorakis et al. (1999) argue that the presence of HSPs may be detrimental when present in the cells for a prolonged period. These findings agree with results from Krebs and Feder (1997), which suggest that the maintenance of high levels of HSP can be harmful with regard to growth, development and survival of Drosophila larvae. In addition, it is suggested that during infection, overexpression of HSP may promote survival of intracellular pathogens (Joslin et al. 1991), otherwise eliminated through apoptosis of the infected cells (Samali and Cotter 1996). Thus, the increased stress tolerance offered by the increased levels of HSP may come at a price, but cells exposed to regular stress (e.g. exercise) do none the less have a definitive need of protection against stress-induced injury. These cells may need to increase the maximal magnitude of their HSP expression either by increasing their maximal protein expression or by (over time) decreasing the baseline level of transcription of the proteins that need to be expressed maximally during stressful events (Thompson et al. 2002). In support of the latter view, we show that after an initial increase in PostExc expression, the PostExc heat shock expression gradually declines with increasing number of repetitions (Fig. 3a–c). Thus, it is a clear attenuation of the HSP response with increasing duration of training and we believe that this attenuation of the HSP response represents an intramuscular adaptation to prolonged training.

In summary, we show that long-term exercise increases HSP72, HSP27 and GRP75 levels in previously untrained non-postural human muscle, and the present study is the first study to show an increase in HSP27 and GRP75 levels in man following training. We also show that there was an effect of training duration on HSP expression in untrained skeletal muscle. Subjects who carried out the most repetitions (elbow extensions) had lower PostExc values than subjects that carried out the fewest repetitions. In addition, subjects with low PreExc levels responded with large changes in the PostExc levels of the HSPs, while subjects with initially high PreExc levels showed a negative development in the PostExc values. Finally, detraining for 8 weeks reduced the PostExc expression of the different HSPs towards PreExc levels. This investigation was performed on a non-postural muscle

and consequently not influenced by daily locomotor activity that might possibly influence the exercise response of the HSPs in postural muscles.

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