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Acute and long-term effects of blood flow restricted training on heat shock proteins and endogenous antioxidant systems

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Abstract

Blood flow restricted exercise (BFRE) with low loads has been demonstrated to induce considerable stress to exercising muscles. Muscle cells have developed a series of defensive systems against exercise-induced stress. However, little is known about acute and long-term effects of BFRE training on these systems. Nine previously untrained females trained low-load BFRE and heavy load strength training (HLS) on separate legs and on separate days to investigate acute and long-term effects on heat shock proteins (HSP) and endogenous antioxidant systems in skeletal muscles. BFRE and HLS increased muscle strength similarly by 12±7 and 12±6%, respectively, after 12 weeks of training. Acutely after the first BFRE and HLS exercise session, αB-crystallin and HSP27 content increased in cytoskeletal structures, accompanied by increased expression of several HSP genes. After 12 weeks of training, this acute HSP response was absent. Basal levels of αB-crystallin, HSP27, HSP70, mnSOD or GPx1 remained unchanged after 12 weeks of training, but HSP27 levels increased in the cytoskeleton. Marked translocation of HSP to cytoskeletal structures at the commencement of training indicates that these structures are highly stressed from BFRE and HLS. However, as the muscle gets used to this type of exercise, this response is abolished.

Keywords: occlusion training, stress proteins, superoxide dismutase, glutathione peroxidase, chaperonin, skeletal muscle, strength training

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Introduction

Blood flow restricted exercise (BFRE) with low loads, also known as KAATSU training, is a popular exercise form which has been demonstrated to induce muscle hypertrophy in magnitudes similar to

traditional heavy load strength training (HLS) (Wernbom *et al.*, 2008). This form of exercise causes the same (or higher) metabolic stress on muscles as HLS (Suga *et al.*, 2009), but without being accompanied by high mechanical forces. In resemblance with other forms of exercise, low load BFRE induces muscle stress and in some cases muscle damage, although this is highly debated (Loenneke *et al.*, 2014). A reliable indicator of muscle damage is alterations in muscle function, i.e. long lasting reductions in force-generating capacity (Paulsen *et al.*, 2012*b*). Substantial reductions in force-generating capacity, and prolonged recovery after BFRE, has previously been reported (Umbel *et al.*, 2009; Wernbom *et al.*, 2012). Furthermore, a more severe form of muscle damage, rhabdomyolysis, is reported in one case-study in an elite athlete, after one bout of low load BFRE (Iversen & Røstad, 2010). On the other hand, no considerable muscle damage after low-load BFRE, or walking with blood flow restriction, indicated by unaltered plasma levels of creatine kinase and myoglobin, is reported in other studies (Takarada *et al.*, 2000; Abe *et al.*, 2006). To our knowledge, the long-term effects of BFRE on systems for handling cellular stress and damage, such as heat shock proteins and proteins involved in the endogenous antioxidant defense systems, has so far not been investigated.

As a physiological response to acute exercise-induced stress, heat shock proteins (HSP), such as αB-crystallin, HSP27 and HSP70, translocate and accumulate in the affected areas within the cell (Koh & Escobedo, 2004; Paulsen *et al.*, 2009; Cumming *et al.*, 2014). The magnitude of this response is related to the type of stress put on the exercised muscles. Muscle actions inducing high-mechanical forces typically induce larger responses than low to moderate force actions (Folkesson *et al.*, 2008; Paulsen *et al.*, 2009). In addition, the total HSP content in exercised muscles seems to increase in response to strength training (Liu *et al.*, 2000, 2004; Gjøvaag & Dahl, 2006; Paulsen *et al.*, 2012*a*), which probably is important for maintaining cell homeostasis. Due to their important function in protection against cell stress, acute HSP responses can be considered as direct markers for cell stress and damage. In a recent study a pronounced acute HSP response, indicated by translocation of small HSPs to cytoskeletal structures, was observed after a single bout of low load BFRE (Cumming *et al.*, 2014). This shows that low load BFRE is highly capable of inducing cell stress and possible damage to cytoskeletal structures.

In addition to the HSPs, cells have developed a network of protective systems against damaging cell stress. To maintain cell homeostasis during periods of increased production of reactive oxygen and nitrogen species (RONS), the cells are highly dependent on antioxidant defense systems such as superoxide dismutases (SOD) and glutathione peroxidases (GPx). As both BFRE and HLS have the potential to induce oxidative damage (Liu *et al.*, 2005; Adachi *et al.*, 2006; Westman *et al.*, 2007), increases in the antioxidant systems could be an important adaptation to prevent oxidative related

63 stress to muscle cells, and consequently increase the tolerance to intense or ischemic exercise, i.e.
64 BFRE.

65 We have previously reported the training induced changes in muscle strength and muscle
66 hypertrophy on the same study population (Ellefsen *et al.*, 2015). The aim of the present study was
67 to investigate i) acute HSP responses in skeletal muscle to BFRE and HLS in untrained and trained
68 (after 12 weeks training) female participants, and to investigate ii) effects of 12 weeks of BFRE and
69 HLS on basal levels of HSPs and antioxidant enzymes in skeletal muscles. We hypothesized that both
70 BFRE and HLS would induce HSP responses acutely after exercise, evident as increased translocation
71 to cytoskeletal structures and increases in mRNA levels in the untrained state. Furthermore, we
72 hypothesized that the acute response would be dampened after 12 weeks of training because of the
73 proposed improvements in exercise tolerance. As a possible mechanism for increased exercise
74 tolerance, we hypothesized that basal skeletal muscle HSP, mnSOD and GPx1 content would be
75 increased after 12 weeks of both BFRE and HLS training.

Methods

Participants

Nine healthy untrained female volunteers (age 22±1 years, height 169±3 cm, body weight 69±5 kg) were recruited. All participants gave written informed consent before entering the study, and were informed about potential risks related to the experiment. The study was approved by the Regional Ethics Committee of South-East Norway and was performed in accordance with the Helsinki Declaration.

Experimental design

The experimental protocols, design and data on muscle strength and hypertrophy have been reported previously (Ellefsen *et al.*, 2015). Briefly, the participants were instructed to perform 12 weeks training of unilateral knee-extension (Technogym, Gambettola, Italy) twice per week. In a randomized order, one leg exercised with low loads and partial blood flow restriction (BFRE), and the other leg exercised with high load resistance exercise (HLS). The partial blood flow restriction was induced by an 18 cm wide pressure cuff (Delfi Medical, Vancouver, Canada) connected to a tourniquet system (Welch Allyn, NY, USA). The BFRE consisted of 5 sets to voluntary failure at 30% of 1 RM with 45 seconds rest between sets. HLS was exercised using the same exercise equipment and three sets of high load resistance exercise were done with free blood flow, with 90 sec rest between sets; each week, one day was performed with moderate-high load (10 RM) and the second day was performed with high load (6 RM). Unilateral knee-extension 1RM for each leg was determined before, after 6 weeks and after the training period using the same unilateral knee-extension apparatus used during the training. The results on muscle strength in the present study include only participants who volunteered for a muscle biopsy. The results published by Ellefsen et al. (2015) included additional participants who did not undergo muscle biopsies.

Muscle sampling and homogenization

Muscle biopsies were sampled from the mid-portion of *m. vastus lateralis* before (pre) and 1 hour after the first (week 0) and the last exercise session (week 12) from both legs and exercise protocols. Resting (control) biopsies was sampled on a separate day than the training biopsy (one hour acute biopsies) to minimize potential influence from the sampling procedure on parameters measured on muscle samples, details about time points have been described previously (Ellefsen *et al.*, 2015). The last exercise bout was conducted after one week of rest to minimize any influence from the previous exercise session. The insertion of repeated biopsies was placed 3 cm proximally from the previous biopsy. Under local anesthesia (Xylocain adrenalin, 10 mg/ml + 5 µg/ml, AstraZeneca PLC, London,

UK), approximately 200 mg (2-3x 50-150 mg) of muscle tissue was obtained with a modified Bergström-technique. Biopsy samples used for homogenization were quickly washed in physiological saline and fat, connective tissue, and blood were discarded. Samples for protein analyses were weighed and snap frozen in isopentane cooled on dry ice. Samples for RNA analyses were weighed and immersed in RNAlater (Ambion, Life technologies, Carlsbad, CA, USA), and treated in accordance with the manufacturer's protocol. All muscle samples were stored at -80^{o} C until homogenization.

For extraction of proteins, about 50 mg of muscle tissue was homogenized in ice cold homogenization buffer (Cat#78510, T-PER/Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA) added protease-phosphatase inhibitors (Cat#1861281, Halt protein and phosphatase inhibitor cocktail, Thermo Scientific) and EDTA (Cat#1861274, Thermo Scientific). For homogenate fractions, ~50 mg of muscle tissue were homogenized and fractionated into a cytosol-, membrane-, nuclear- and cytoskeletal fractions using a commercial fractionation kit (ProteoExtract Subcellular Proteo Extraction Kit, Cat#539790, Calbiochem, EMD Biosciences, Schwalbach, Germany) in accordance to the manufacturer's procedures. Protein concentrations were assessed with a commercial kit (BioRad DC protein micro plate assay, Cat#0113, Cat#0114, Cat#0115, Bio-Rad, Hercules, CA, USA), a filter photometer (Expert 96, ASYS Hitech, UK) and the provided software (Kim, ver. 5.45.0.1, Daniel Kittrich). The average protein yield of the cytosolic fraction was 1.1±0.4 mg and 2.9±0.6 mg in the cytoskeletal fraction, and was stable over all time-points. For extraction of total RNA, muscle tissue was homogenized in TRIzol reagent (Cat# 15596, Invitrogen, Carlsbad, CA, USA), as previously described (Ellefsen *et al.*, 2008). Prior to extraction, care was taken to remove all remnants of RNA*later* from the muscle samples. RNA quantities were obtained using a spectrophotometer (Nanodrop 2000c UV-Vis, Thermo Scientific).

Immunoblot and ELISA

Extracted proteins were analyzed in duplicates (mean values were used for statistical analyses) using the dry western blot technique (iBlot dry blotting system, Cat#IB1001, Invitrogen). Equal amount of protein was separated by 4-12% SDS gradient gels under denaturized conditions. Proteins were transferred onto PVDF-membranes (iBlot Gel transfer stacks, Cat#IB4010, Invitrogen), before being blocked in a 5% fat free skimmed milk and TBS-t solution (TBS, Cat#170-6435, Bio-Rad; 0.1% Tween-20, Cat#437082Q, VWR International, Radnor, PA, USA; Skim milk, Cat#1.15363, Merck, Darmstadt, Germany). Blocked membranes were incubated with antibodies against HSP70 (mouse-anti HSP70, Cat#SPA-810, Enzo Life Sciences, NY, USA), αB-crystallin (mouse-anti αB-crystallin, Cat#SPA-222, Enzo Life Sciences), GPx1 (Cat#ab22604, Abcam, Cambridge, UK) or mnSOD (Cat#ab16956, Abcam), and an appropriate secondary antibody (goat anti-mouse, Cat#31430, Thermo Scientific; Goat anti-rabbit,

Cat#7074, Cell Signaling Technology, MA, USA). All antibodies were diluted in a 1% fat free skimmed milk and TBS-t solution. Bands were visualized using an HRP-detection system (Super Signal West Dura Extended Duration Substrate, Cat#34076, Thermo Scientific, IL, USA). Chemiluminescence was measured using a CCD image sensor (Image Station 2000R or Image Station 4000R, Eastman Kodak Inc., Rochester, NY, USA) and band intensities were calculated with the Carestream molecular imaging software (Carestream Health Inc., Rochester, NY, USA). Since the cytoskeletal fraction usually do not contain αB-crystallin or HSP70, cytoskeletal αB-crystallin and HSP70 was normalised against pre cytosolic levels to show subcellular translocation.

HSP27 in the cytosolic and cytoskeletal fractions was measured with an in-house-made double-antibody sandwich ELISA using a monoclonal capture antibody against HSP27 as described previously (Cumming *et al.*, 2014)

qRT-PCR

Gene analyses were conducted as previously described (Ellefsen *et al.*, 2015). Briefly, reverse transcription was performed on 500 ng total RNA using Superscript III Reverse Transcriptase (Cat#18080-085, Invitrogen), primed with both random hexamers (Cat#8080127, Ambion, Life technologies) and oligo(dT) (Cat#18418-020, Ambion), in accordance to the manufacturer's procedures. cDNA synthesis was performed in duplicates for each sample.

qRT-PCR was performed on 1:30 ilutions of cDNA using PerfeCTa SYBR Green FastMix (Cat#95072, Quanta Biosciences Inc., Gaithersburg, MD, USA) or SYBR select master mix (Cat#4472920, Life technologies; only HSPA1A and HSPA1B mRNA analyses) and the 7500 Fast Real-Time PCR System (Applied Biosystems, Life technologies), according to manufacturer's protocol. Ct was calculated using the 7500 Fast Real-Time PCR System software; priming efficiencies (E) were calculated using the LinRegPCR software (Ruijter *et al.*, 2009). For final calculations of target gene expression, mean priming efficiencies were utilized, calculated separately for each primer pair. Average E and Ct values for each of the primer pairs are listed in Table 1. Mean values of peptidylprolyl isomerase A (PPIA) and ribosomal protein L32 (RPL32) were used as normalization genes as this has been found to be unaffected by 12 weeks of heavy strength training (Ellefsen *et al.*, 2014, 2015).

Statistics

All values are presented as means ± standard deviations (SD). All data were tested for normality with a Shapiro-Wilk normality test. If data were not normally distributed, log-transformation was applied to achieve normality before further analyses. A two-way ANOVA was used to evaluate the acute and long term effects of training and type of exercise, and a Holm-Sidak multiple comparisons test was

applied for post hoc analyses. Paired t-test was used to evaluate differences between exercise responses in untrained (first exercise bout) and trained (last training bout) skeletal muscles. Figures display individual data points and means and standard deviations. The level of significance was set to $P \leq 0.05$. Graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses.

Results

After 12 weeks of training, 1RM increased by 12±7 and 12±6% in the BFRE and HLS leg, respectively (figure 1), with no differences between exercise interventions.

Acute heat shock protein responses

Untrained state. Acutely after the first exercise session (week 0), αB-crystallin decreased in the cytosolic fraction after both BFRE and HLS (-37±28 and -38±23%, respectively; *P*<0.001; figure 2A), with no differences between exercises. The decrease in the cytosolic αB-crystallin was accompanied by an increase in the cytoskeletal fraction after both BFRE and HLS (8±7 and 5±3% [cytoskeletal levels are normalized against cytosolic levels]), respectively; *P*<0.001; figure 2B), with no differences between exercises. Because the cytoskeletal fraction contains about three times more protein than the cytosolic fraction, the 5-8% increase in cytoskeletal αB-crystallin is close to matching the 37-38% decrease in cytosolic αB-crystallin. Cytosolic HSP27 levels remained stable 1 hour after the first exercise session (figure 2C), whereas cytoskeletal HSP27 levels increased by 55.3±30.1 and 45.9±29.5 ng HSP27 for BFRE and HLS, respectively (corresponding to ˜9% of the cytosolic content; *P*<0.001; figure 2D), with no differences between exercises. HSP70 levels were stable 1 hour after the first exercise bout, with no changes in the cytosolic- or cytoskeletal fraction (figure 2E-F).

Trained state. After 12 weeks of training, the acute αB-crystallin response was absent, with no changes in cytosolic or cytoskeletal αB-crystallin levels following neither BFRE nor HLS (figure 2A-B). Cytosolic HSP27 levels were decreased after HLS, but not after BFRE (figure 2C; *P*=0.030). Despite a reduction in cytosolic HSP27 levels, no changes were observed in cytoskeletal HSP27 levels (figure 2D). An increase was observed in cytosolic HSP70 levels 1 hour after BFRE in the trained state (45±54%; *P*=0.044; figure 2E).

Acute gene expression responses

Untrained state. One hour after the first exercise bout, *CRYAB*, *HSPB1*, *HSPA1A*, *HSPA1B* and *HSP90AA1* mRNA increased after both BFRE and HLS (*P*<0.05; figure 3A-B, F-H), with no differences between exercises. *HSPB2*, *HSPB3*, *HSPD1, SOD1*, *SOD2* and *GPx1* mRNA remained unchanged compared to pre exercise mRNA expression (figure 3C-E, 4).

Trained state. Similar to in the untrained state, *CRYAB*, *HSPB1* and *HSP90AA1* mRNA increased one hour after the last exercise bout (after 12 weeks of training) ($P<0.05$; figure 3A-B, H) after both BFRE and HLS, with no differences between exercises. *HSPB2*, *HSPB3*, *HSPD1,* HSPA1A, HSPA1B, SOD1, *SOD2* and *GPx1* mRNA did not change compared to pre exercise mRNA expression (figure 3C-G, 4).

Chronic training adaptations

Basal protein levels of αB-crystallin, HSP27, HSP70, mnSOD and GPx1 did not change over 12 weeks of BFRE or HLS training (figure 5 and 6). Further analyses revealed that the participants had higher resting cytoskeletal HSP27 levels (basal pre levels) in both legs after the training intervention compared to before the training intervention (BFRE: 269±471%. HLS: 61±157%; $P=0.045$; figure 2D).

Basal mRNA expression of *CRYAB*, *HSPB2*, *HSPA1A* and *HSPA1B* mRNA levels increased, and basal *SOD2* and *GPx1* mRNA levels decreased after 12 weeks of BFRE and HLS ($P\leq0.05$; figure 7A, C, F-G and 8B-C), with no differences between exercises. HSPB1 mRNA levels adapted in opposite direction in the two training modes (interaction effect; $P=0,028$; figure 7B), with the resting mRNA levels increasing in the BFRE trained leg and decreasing in the HLS leg. Basal expression of HSPB3, *HSPD1*, *HSP90AA1* and *SOD1* remained unchanged after 12 weeks of training (figure 7D-E, H, 8A).

Discussion

The present study investigated aspects of the acute stress response to BFRE and to HLS at the onset and at the end of a 12-week training period. The main results showed that both BFRE and HLS induced a pronounced HSP and HSP mRNA response in the untrained state. However, in the trained state (after 12-weeks of BFRE or HLS) the acute stress response to the standardized exercise session was absent, whereas the acute HSP mRNA response was generally intact. The increased exercise tolerance to both protocols after 12-weeks of training could not be explained by changes in basal protein levels of αB-crystallin, HSP27, HSP70, mnSOD or GPx1.

Heat shock proteins

Acute responses

We have previously demonstrated that a single bout of low load BFRE acutely increases αB-crystallin, HSP27 and HSP70 levels in cytoskeletal structures (Cumming *et al.*, 2014). In the present study, we observed similar acute translocation of αB-crystallin and HSP27 to cytoskeletal structures after BFRE. Interestingly, similar responses were also observed after HLS. This indicates that BFRE induces myofibrillar stress responses similar to what is found after HLS. In support of this, no differences were observed in the acute changes in mRNA expression between the two exercises. While muscle

damage is often observed after HLS (Gibala *et al.*, 1995), it is commonly believed that BFRE induces less cellular stress and consequently do not induce muscle damage (Loenneke *et al.*, 2014). This notion might be true when the muscles are accustomed to BFRE training, but muscle damage most likely occurs when the muscles are not accustomed to this type of exercise, and most likely if the exercise is done to voluntary failure. It is tempting to speculate that the HSP translocation to cytoskeletal structures was induced by different cellular stressors in the two exercise protocols. Indeed, HSPs have been shown to translocate and accumulate in myofibrillar structures after high load strength exercises (Paulsen *et al.*, 2007; Folkesson *et al.*, 2008), arguably involving high mechanical forces as the main stressor. HSPs have also been shown to translocate after ischemic insults (Yoshida *et al.*, 1999; Armstrong *et al.*, 1999; Golenhofen *et al.*, 2004). Since BFRE typically involve low mechanical forces, but considerable time under ischemic conditions (Kacin & Strazar, 2011; Karabulut *et al.*, 2014), it is plausible that translocation to cytoskeletal structures is related to ischemia rather than the stressors that occurs during HLS. Indeed, Cumming et al. (2014) found BFRE to induce HSP responses to be more pronounced in type 1 than in type 2 fibers. Contrary, Folkesson et al. (2008) showed that HLS induced an HSP27 response mainly in the type 2 fibers. Thus, we believe that the two exercise protocols not only induce different types of stressors, but also affect the two fiber types differently. Unfortunately, we were not able to address the fiber type specific HSP response in this study.

Chronic adaptations to training

Interestingly, the acute HSP responses observed by translocation of HSP to cytoskeletal structures after the initial exercise session (in week 0 of the training intervention), were not observed after the final bout in week 12. Initially, we hypothesized that this indicates improved exercise tolerance related to increased basal levels of HSPs and endogenous antioxidant enzymes. In contrast to our hypothesis, 12 weeks of BFRE training did not increase basal levels of HSP or antioxidant enzymes. No change in HSP protein levels was unexpected because of the pronounced gene expression of some of the HSP genes after the first exercise session. Normally, such a response indicates increased need for HSPs, and increased HSP levels have previously been found after HLS (Liu *et al.*, 2000, 2004; Gjøvaag & Dahl, 2006; Paulsen *et al.*, 2012*a*). However, we must interpret the HSP mRNA data with caution, as it has been reported that some HSP mRNAs (especially the HSPA1A and/or HSPA1B) might be affected by the sampling procedure itself rather than the exercise performed (Vissing *et al.*, 2005). However, we report stable mRNA levels even with repeated biopsy sampling. Previously, we even found HSP70 protein levels to be elevated for several days after a bout of low load BFRE (Cumming *et al.*, 2014). Increased HSP levels found after several training studies could therefore be a result of the last exercise bout rather than chronic increased HSP levels.

Interestingly, we observed increased basal HSP27 levels in cytoskeletal structures after 12 weeks of training in both legs. This indicates that these structures had increased demands of HSP27 for further recovery. Increased levels of HSP27 in these structures could not fully explain some of the increased exercise tolerance observed after the last exercise (indicated by acute HSP responses). Indeed, studies overexpressing HSP70 10-20 times over normal will increase the general stress tolerance (Mcardle *et al.*, 2003), but these levels are much higher than what was observed in the present study. Nevertheless, we cannot exclude the possibility that the small increase in the cytoskeletal HSP27 will protect vulnerable structures in the cytoskeleton and thus improve exercise tolerance. Additionally, adaptations in e.g. the extra cellular matrix (Mackey *et al.*, 2011) or the inflammatory response (Lapointe *et al.*, 2002; Hubal *et al.*, 2008) might have increased the tolerance to exercise and reducing the exercise stress. Furthermore, the biopsy sampling in the present study was done in one direction (proximal to the previous sampling site). We could speculate that regional differences within the muscle is responsible for the observed differences in cellular phenotype after 12 weeks of training. However, Paulsen et al. (2007) did not observe any HSP accumulation in the control muscle after repeated biopsy sampling similar to our sampling procedure.

A clear difference between the studies reporting increased HSP content and the present study is that we only included female participants. Since sex-specific differences in HSP content in response to training has previously been suggested, it is tempting to point to this as a potential explanation behind these seemingly conflicting results in basal HSP content. Indeed, male rats display higher content of HSP27, HSP70 and HSP90 in slow twitch muscles and higher content of HSP60 in fast twitch muscles compared to female rats (Voss *et al.*, 2003). Further, Morton et al. (2009) observed that females did not increase αB-crystallin and HSP60 content to similar extents as males in response to endurance training. However, Gjøvaag and Dahl (2006) did not observe any sex differences in HSP27 or HSP70 content after strength training. Overall, we cannot exclude the possibility that sex has influenced adaptations in HSP levels.

In addition to sex-related aspects, it is possible that two training sessions per week was not sufficient to increase HSP levels over time. Nevertheless, *CRYAB*, *HSPB1, HSPA1A and HSPA1B* mRNA expression increased acutely after exercise, both in the untrained and trained state. Thus, both types of exercises were performed with intensities sufficient to induce expression of these HSPs, but the time between exercises (3-4 days) might have allowed complete normalization between sessions, and consequently prevented the possibility for cumulative effects during the training period.

Endogenous antioxidants

In addition to the HSPs, the endogenous antioxidant systems are also important for maintenance of cell homeostasis (Powers & Jackson, 2008). We did however not observe any acute increases in *SOD1*, *SOD2* or *GPx1* mRNA expression after a single bout of BFRE or HLS, neither in the untrained nor the trained state. Contrary, we observed decreased resting mRNA levels of *SOD2* and *GPx1* mRNA after the training period. This was supported by the lack of change in protein levels of GPx1 and mnSOD during 12 weeks of training. It is therefore plausible to suggest that the concentration of these enzymes was sufficient to deal with the RONS produced during the training sessions.

Exercises that rely heavily on anaerobic energy supply, such as strength and sprint exercises, has a great potential to produce RONS (Morales-Alamo & Calbet, 2014), and thus potentially induce oxidative stress; a stimulus for the expression of antioxidant enzymes (Radak *et al.*, 2001). Nonetheless, few studies investigating the effects of strength training exists, and they show somewhat inconsistent results (Ryan *et al.*, 2010; Scheffer *et al.*, 2012). Furthermore, blood flow restriction has the potential to induce oxidative damage if the blood flow is restricted for longer periods (Adachi *et al.*, 2006; Westman *et al.*, 2007). Despite this, only a few studies have successfully measured markers for oxidative damage after BFRE, with equivocal results (Takarada *et al.*, 2000; Goldfarb *et al.*, 2008). However, these indirect markers of oxidative damage/stress were measured in blood samples after exercise with relative small muscle mass. Consequently, the likelihood of finding changes in these markers in blood sampled from an arm vein would be small. The most likely explanation for our results is that the exercises per se did not result in considerable alterations in RONS and thereby no real stimuli for increases in GPx1 or mnSOD were present.

Conclusion

The present study demonstrated similar acute translocation of αB-crystallin and HSP27 to the cytoskeletal structures in the untrained state after BFRE and HLS. Notably, the acute translocation of αB-crystallin and HSP27 was abolished in the trained state (after 12 weeks training). This indicates adaptations in the muscle that make it more resistant against stressful insults occurring during BFRE and HLS. The improved exercise tolerance could, however, not be explained by elevated basal levels of HSPs or endogenous antioxidant enzymes, because both systems were unaltered after 12 weeks of BFRE and HLS training. However, increased cytoskeletal HSP27 levels may have contributed to the improved exercise tolerance.

Perspectives

Improved capacity to deal with exercise-induced stress seems to be an important adaptation to strength training. Based on our results, 12 weeks of BFRE or HLS does not increase HSP levels or

endogenous antioxidant enzymes in exercised skeletal muscles of previously untrained females. In addition, we demonstrate that the acute HSP response after BFRE and HLS occurs only in the untrained state, whereas trained muscles does not display the same responses. Taken together, the exercise induced HSP response and increases in basal levels of HSP and endogenous antioxidant enzymes reported previously might be the response of high levels of cellular stress rather than a chronic adaptation to strength training. This suggests that the improved stress tolerance in response to training is mainly caused by other adaptations in skeletal muscles. Further investigations in humans should therefore be conducted to understand adaptations in these systems in response to strength training and to identify other adaptations that improves stress tolerance.

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Figure Legends

Figure 1. Individual percentage changes (%-change) in maximum strength (1RM) in knee extension after 12 weeks of BFRE (left) and HLS (right) training. Stippled line indicates baseline levels (0%).

Figure 2. Acute changes in cytosolic αB-crystallin (**A**), cytoskeletal αB-crystallin (**B**), cytosolic HSP27 (**C**), cytoskeletal HSP27 (**D**), cytosolic HSP70 (**E**) and cytoskeletal HSP70 (**F**) protein levels, before (left side on graphs) and after 12 weeks of BFRE or HLS training (right side on graphs). Lower panels show immunoblots for αB-crystallin and HSP70 for both cytosolic (C) and cytoskeletal (CSK) fraction. Note that the immunoblots are put in order to fit this graph. Immunoblot data from the cytosolic fraction is expressed as percentage change from pre exercise levels. Immunoblot data from the cytoskeletal fraction is normalized against pre cytosolic levels and expressed relative to cytosolic protein levels. HSP27 was measured with ELISA and expressed as ng HSP27 per mg protein. Stippled line indicates baseline levels (0 or 100%). *: different between exercises (Interaction effect; $P<0.05$).

Figure 3. Acute changes in mRNA expression for *CRYAB* (**A**), *HSPB1* (**B**), *HSPB2* (**C**), *HSPB3* (**D**), *HSPD1* (**E**), *HSPA1A* (**F**), *HSPA1B* (**G**) and HSP*90AA1* (**H**), before (left side on graphs) and after 12 weeks of BFRE or HLS training (right side on graphs). mRNA expression is expressed as ratio between post and pre expression. Stippled line indicates ratio of 1 (equal expression between pre and post).

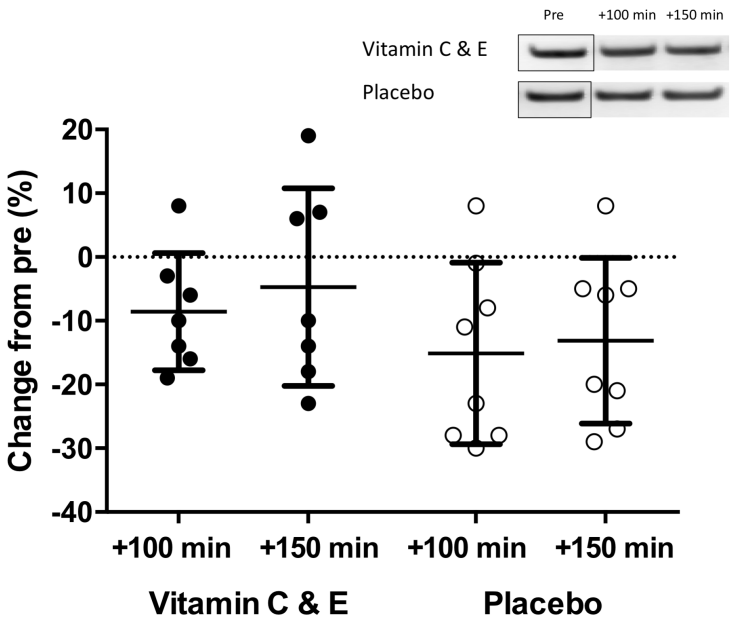
Figure 4. Acute changes in mRNA expression for *SOD1* (**A**), *SOD2* (**B**) and *GPx1* (**C**)), before (left side on graphs) and after 12 weeks of BFRE or HLS training (right side on graphs). mRNA expression is expressed as ratio between post and pre expression. Stippled line indicates ratio of 1 (equal expression between pre and post).

Figure 5. Changes in αB-crystallin (**A**), HSP27 (**B**) and HSP70 (**C**) protein levels after 12 weeks of BFRE and HLS training. Panels show immunoblots for αB-crystallin and HSP70 pre and after 12 weeks of BFRE or HLS training. Stippled line indicates baseline levels (0%).

Figure 6. Changes in mnSOD (**A**) and GPx1 (**B**) protein levels after 12 weeks of BFRE and HLS training. Panels show immunoblots for mnSOD and GPx1 pre and after 12 weeks of BFRE or HLS training. Stippled line indicates baseline levels (0%).

Figure 7. Changes in basal mRNA expression for *CRYAB* (**A**), *HSPB1* (**B**), *HSPB2* (**C**), *HSPB3* (**D**), *HSPD1* (**E**), *HSPA1A* (**F**), *HSPA1B* (**G**) and HSP90AA1 (**H**) after 12 weeks of BFRE or HLS training. mRNA expression is expressed as ratio between post and pre expression. Stippled line indicates ratio of 1 (equal expression between pre and post). *: different between exercises (Interaction effect; $P<0.05$).

Figure 8. Changes in resting mRNA expression of *SOD1* (**A**), *SOD2* (**B**) and *GPx1* (**C**) after 12 weeks of BFRE or HLS training. mRNA expression is expressed as ratio between post and pre expression. Stippled line indicates ratio of 1 (equal expression between pre and post).



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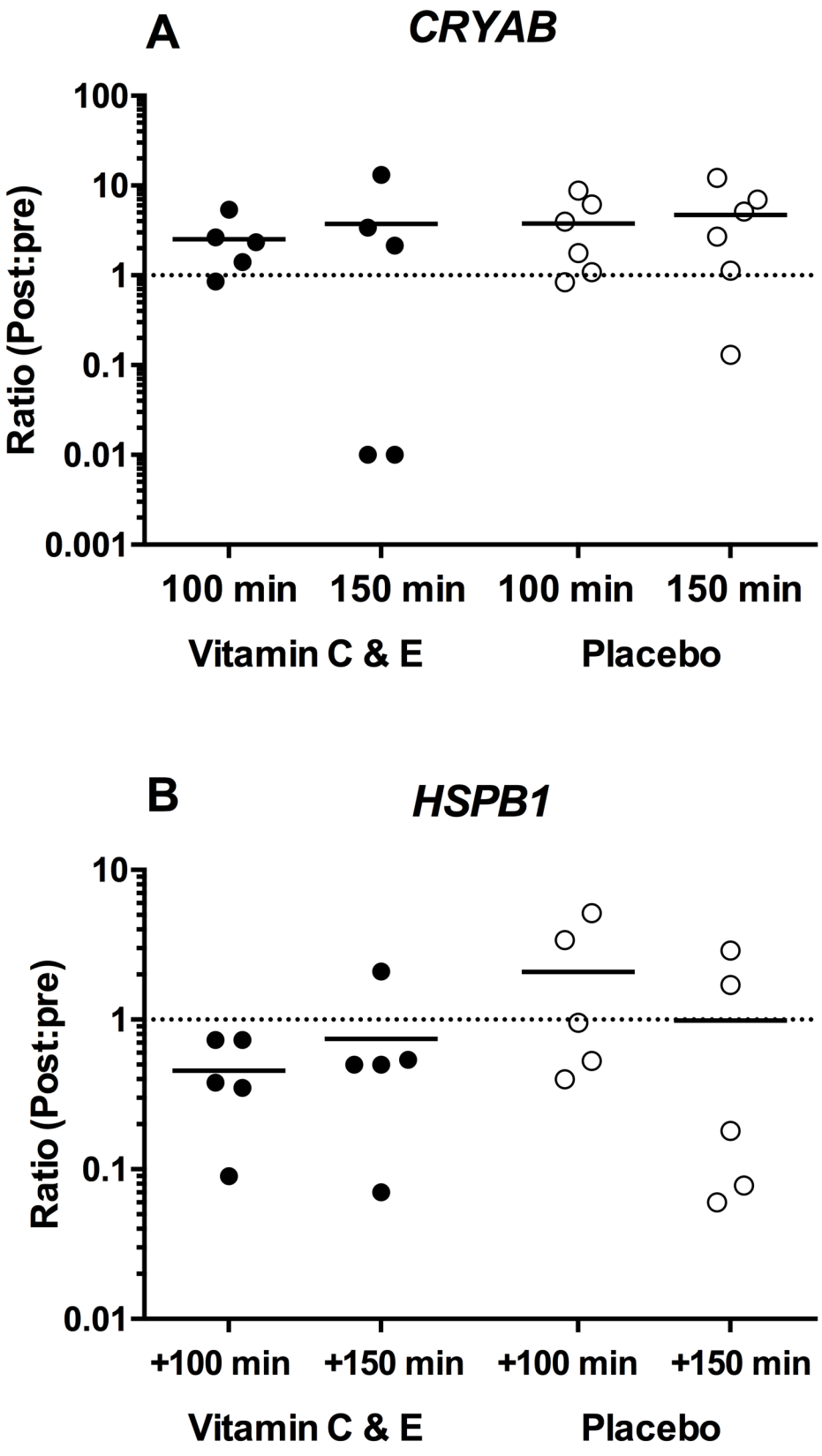
506 Figure 1



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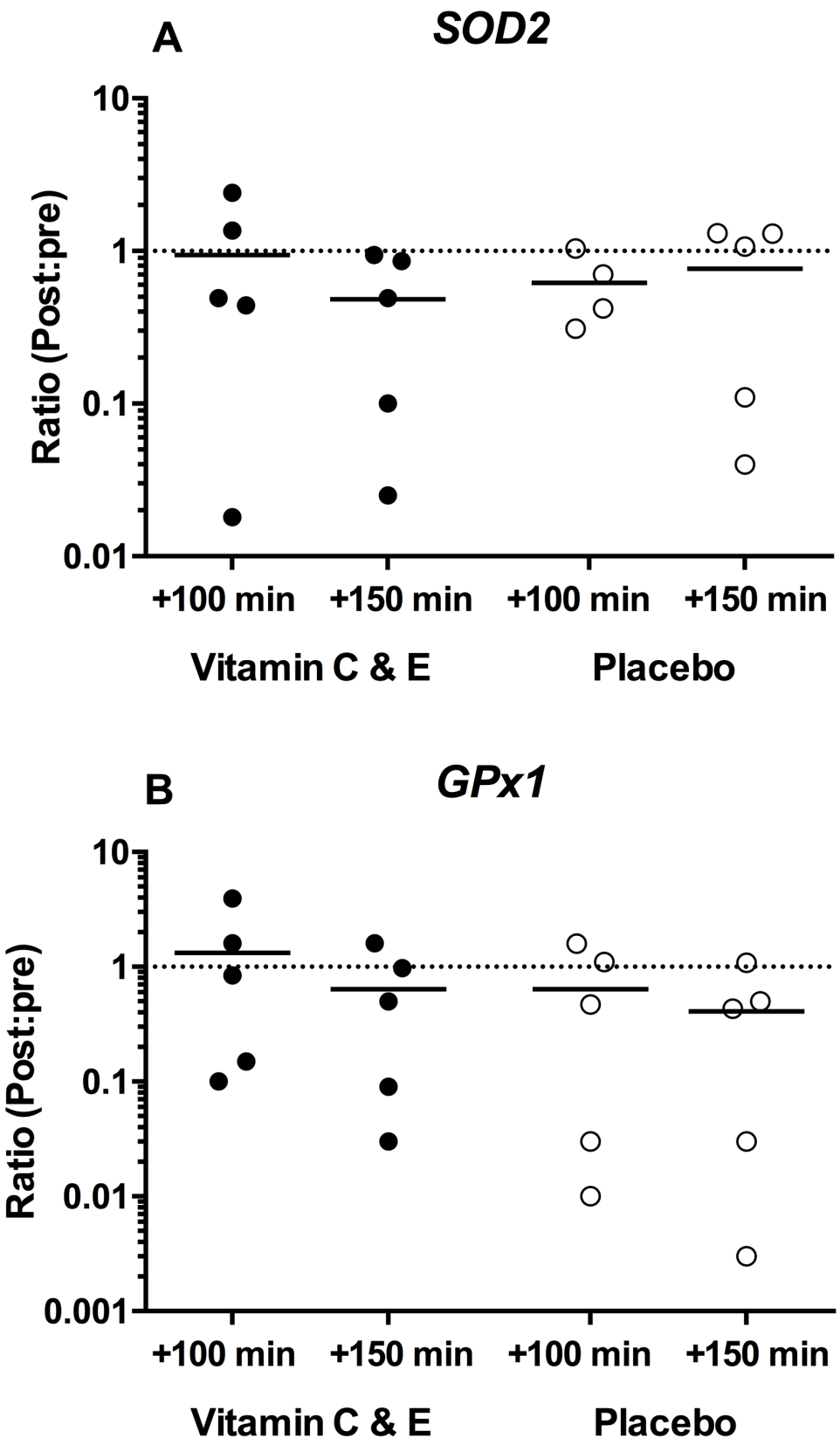

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510 Figure 3

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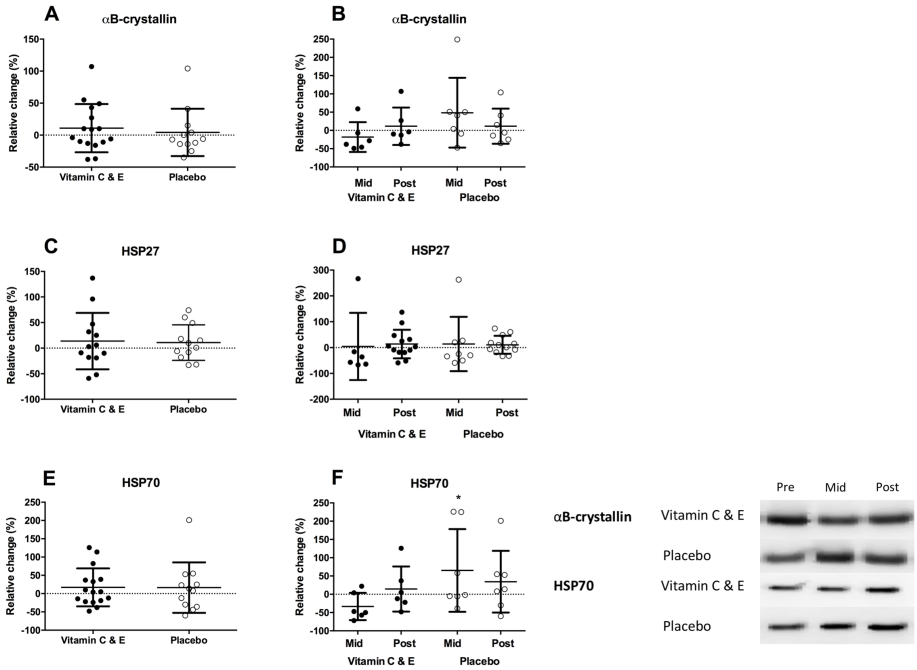

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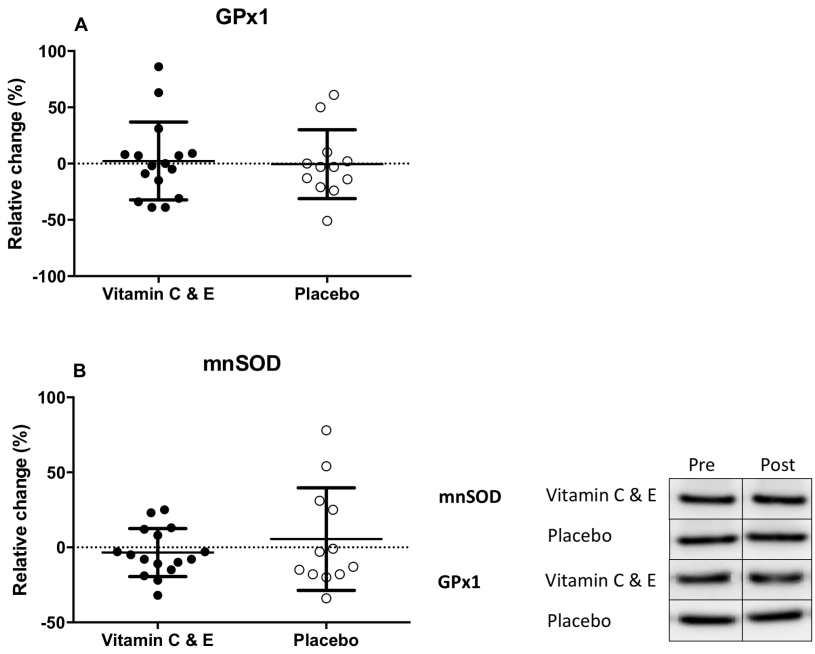
513 Figure 4



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515 Figure 5

Table 1. Human primer sequences used for RT-qPCR

Gene name (accession no.)	**Sense**	**Antisense**
CRYAB (NM_001885)	GTCAACCTGGATGTGAAGCA	TTTTCCATGCACCTCAATCA
HSPB1 (NM_001540)	GGACGAGCTGACGGTCAAG	AGCGTGTATTTCCGCGTGA
SOD2 (NM_000636)	CCCTGGAACCTCACATCAAC	GGTGACGTTCAGGTTGTTCA
GPx1 (NM_000581)	ACGATGTTGCCTGGAACTTT	TCGATGTCAATGGTCTGGAA

The genes listed encodes to following proteins: *CRYAB* = αB-crystallin; *HSPB1* = HSP27 protein 1; *SOD2* = superoxide dismutase 2 or mnSOD; *GPx1* = glutathione peroxidase 1.

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