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Heat shock proteins and endogenous antioxidants in skeletal muscle – Acute responses to exercise and adaptations to training

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An Expert is a person who has made all the mistakes that can be made in a very narrow field

-Niels Bohr

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List of papers

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Cumming, K. T., S., Ellefsen, S. B. R. Rønnestad, and T. Raastad. Acute and long-term effects of low load blood flow restricted resistance training on heat shock proteins and endogenous antioxidant systems in skeletal muscles of untrained females.

Cumming, K. T., T. Raastad, G. Holden, N. E. Bastani, D. Schneeberger, M. P. Paronetto, N. Mercatelli, H. N. Østgaard, I. Ugelstad, D. Caporossi, R. Blomhoff, and G. Paulsen. (2014). Effects of vitamin C and E supplementation on endogenous antioxidant systems and heat shock proteins in response to endurance training. Physiol Rep 2(9):e12142.

Cumming, K. T., T. Raastad, A. Sørstrøm, M. P. Paronetto, N. Mercatelli, D. Caporossi, and G. Paulsen. Vitamin C and E supplementation does not alter heat shock proteins or endogenous antioxidants in skeletal muscles after 12 weeks of strength training.

Introduction

Introduction to the stress responses in skeletal muscle

The purpose of the current introduction is to give a short overview of the muscle cellular defense systems, with special emphasis on the heat shock proteins and antioxidant systems.

Muscle cells are constantly exposed to stressful insults when performing its "call in life" – to contract. When the capacities (force and endurance) of a muscle are challenged, alterations in cell homeostasis lead to decreased function; e.g. muscle fatigue. While homeostatic imbalance most often is rapidly restored, extremely intensive, high-force exercise may induce myofibrillar disruptions and subsequent inflammation and even cell death (necrosis) (Allen *et al.*, 2008; Paulsen *et al.*, 2012*b*). However, as an outcome to systematic and repeatedly stresses when training, the muscle adapt to improve function and become better to meet future demands. These training adaptations are those responsible for increased muscle strength (e.g. muscle hypertrophy) or those responsible for increased endurance (e.g. increased mitochondrial content). These adaptive capabilities are referred to as muscle plasticity. Muscle plasticity can be inferred after a single exercise session and just a few sessions are enough to measure morphological and functional changes (Seynnes *et al.*, 2007). This makes the skeletal muscle such a remarkable and fascinating tissue to study.

During exercise the muscle is exposed to mechanical stress (Lauritzen *et al.*, 2009), periods of ischemia (Sjøgaard *et al.*, 1988) and formation of reactive oxygen and nitrogen species/free radicals (RONS) increase (Davies *et al.*, 1982); all which have the potential to increase cell stress and induce damage (as discussed later). As the muscle gets more adapted (e.g. stronger with strength training), it gets more suited to withstand the exercise-induced stress put on the muscle. Even though the muscle cell is prepared and capable to tackle these stresses, alterations in the stable cell environment could still take place if the stress gets to large from very high intensity exercise. As a response to cell stress, the cells activate and adjust its defense systems to ensure cell homeostasis and prepare the repair processes. If the muscle cell is frequently inflicted with high levels of stress these defense systems will increase in magnitude. Thus, these stresses are important for further adaptations, and it is important to control, not block, these stresses. Although exercise induces a range of different stresses, the knowledge on how different stresses individually affect the defense systems is still incomplete and limited.

A typical stress response to exercise is a general increase in heat shock protein (HSP) synthesis and immediate translocation to stressed and damaged areas within the cell. This can occur after mechanical tears in the sarcomeres (Koh & Escobedo, 2004; Paulsen *et al.*, 2009), after ischemic conditions (Yoshida *et al.*, 1999; Armstrong *et al.*, 1999; Golenhofen *et al.*, 2004), and increased RONS production (Fittipaldi *et al.*, 2014). The HSPs are not the only defense system against RONS. The endogenous antioxidant system, which is the cells own antioxidants, is the main defense against RONS. These antioxidants have the aim to prevent RONS-induced damage by neutralizing RONS, either by enzymatic reactions or by electron donation. Hence, due to its important defensive role, alterations in the HSP and the antioxidant systems are very important adaptations to training. Details about the HSPs and the endogenous antioxidants will be presented later.

As mentioned above, synthesis of HSPs is increased in stressed cells. The upregulation is controlled by several transcription factors, but the heat shock factor 1 (HSF1) is considered the main inducer.

Somewhat simplified: in unstressed cells, HSF1 is bound to HSPs (HSP70 and HSP90) and in an inactive form, but upon stress conditions, the HSPs are released and HSF1 is free to translocate to the nuclei and bind to heat shock elements (HSE). HSE is the binding site located at HSP genes. This makes HSF1 a direct stress sensor that reacts to stresses like increased RONS concentration (Ahn & Thiele, 2003). Activation of HSP genes can also be regulated and co-regulated by other transcription factors like NFκB[1], CREB[2] and NF-Y[3] (Tranter *et al.*, 2010; Sasi *et al.*, 2014). Some of these transcription factors seem to respond to different types of stress; for instance, NFκB seems to respond to ischemia, while NF-Y does not (Sasi *et al.*, 2014). This coordinated regulation might be important for the magnitude of expression, or it can be important for nuanced stress sensing.

The Nrf2[4] is considered to be the master regulator of the antioxidant genes (Lewis *et al.*, 2010; Kansanen *et al.*, 2013). In unstressed cells, Nrf2 is bound to Keap1[5], but upon RONS-stimulation Nrf2 is released in order to translocate to promoter regions on antioxidant genes. Several antioxidants genes also contain binding sites for NFκB and AP-1[6], which are sensitive to RONS (Kabe *et al.*, 2005; Ji, 2007; George *et al.*, 2012). Due to the vast number of different endogenous antioxidants it is most likely that the upregulation of these genes are controlled and co-controlled by several various transcription factors.

The NFκB-family consist of five different transcription factors (p50/p105, p52/p100, p65/RelA, Rel B, c-Rel) (Kandarian & Jackman, 2006). NFκB plays a double role in the cell, where it can act as signal for "negative" pathways that for instance induce apoptosis and inflammation, or it can play in "positive" pathways which play an important role in development and differentiation (Perkins, 2000; Kandarian & Jackman, 2006). NFκB is considered the "first in line" of all the stress signal pathways. Hence, NFκB is regarded as a key player in cell stress research.

Due to the dual roles as transcription factors, it is believed that members of the NFκB-family have different roles in controlling gene expression (Häcker & Karin, 2006). The NFκB p65 is, however, considered the most important of the NFκBs in muscle and in the induction of endogenous antioxidants and HSPs (Wan *et al.*, 1994; Hollander *et al.*, 2001; Zhou *et al.*, 2001; Ji *et al.*, 2004; Tranter *et al.*, 2010; George *et al.*, 2012; Sasi *et al.*, 2014).

[1] Nuclear factor kappa B; transcription factor which is important for the stress response and protection against stress and injury
[2] cAMP response element-binding protein
[3] Nuclear transcription factor Y
[4] Nuclear factor erythroid 2-related factor
[5] Kelch-like ECH-associated protein 1
[6] Activator protein 1

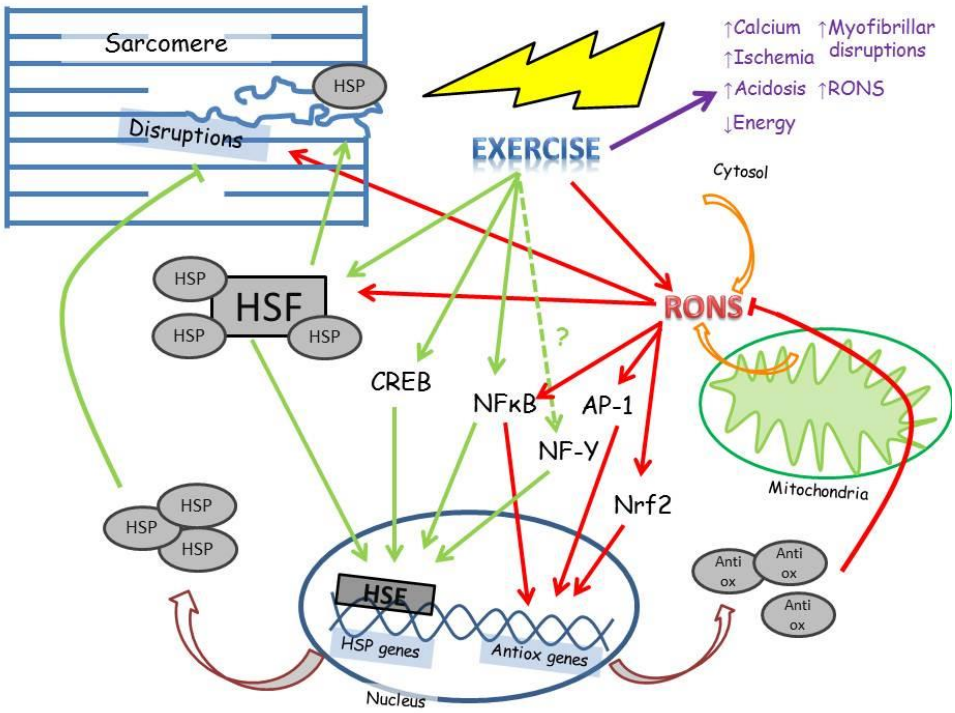

Figure 1. Schematic illustration of the stress responses described above. Exercise induces a range of stresses in the muscle cell, which leads to e.g. increased disruptions in the contractile apparatus, and increased RONS formation. These stresses lead to activation of several stress pathways which eventually increase gene expression of heat shock proteins and antioxidant enzymes. HSP, heat shock proteins; HSF, heat shock factor; HSE, heat shock elements; Antiox, antioxidant enzymes; RONS, reactive oxygen and nitrogen species; CREB, cAMP response element-binding protein; NFκB, nuclear factor kappa B; NF-Y, nuclear transcription factor Y; AP-1, activator protein 1; Nrf2, nuclear factor erythroid 2-related factor.

Discovery of the heat shock protein response

Once upon a time[7], more precise in the early 60s. A young scientist sits in a dark lab lit up by the lights of his laboratory lamp. The sounds of *Love Me Do* are played from the little brown radio in the corner of the lab. He doesn't know that tomorrow he will make a revolutionary discovery that will be a big step in the scientific world of cell stress. The young scientist, named Ferruccio Ritossa, was tired that night. His supervisor, Professor Buzzati, had stepped up the activity after the recent discovery of messenger RNA, and the motivation was very high to make new discoveries in the field of genetics. The sounds of *Love Me Do* continues to come from the little brown radio. "*Im really tired of that song"* a tired Ferruccio says to himself before turning the radio off. "*Now I'm going home for some sleep*". He looks quickly over at his incubator, before cleaning his desk. What Ferruccio didn't see was that one of his colleges had accidentally increased the temperature in his incubator.

[7] Based on true story with fictional episodes added

What happened next was the discovery of the heat shock protein response. What met Ferruccio the next day was fascinating. After the heating accident, his *Drosophilia* was exposed to something referred to as heat shock. This prompted increased gene expression in the *Drosophilia,* observed as chromosomal puffing (Ritossa, 1962). This is actually one of the clearest observations of changes in genes caused by environmental factors. The chromosomal puffing was later identified be related to the expression of a group of proteins named heat shock proteins (HSP) (Tissières *et al.*, 1974). These proteins have distinct roles as chaperone proteins in the cell, and have distinct roles in both unstressed and stressed cells, as discribed in the following sections.

Biological role of heat shock proteins in unstressed and stressed cells

The heat shock proteins have many roles in cells, and each class of HSPs have unique roles and properties to ensure cell homeostasis, and they are important for cells to survive upon stressful insults (Noble *et al.*, 2008).

In unstressed cells, heat shock proteins have been identified to act as proteins to ensure normal cell function. Their roles mainly involve chaperone activities to make sure of correct folding of newly synthesised proteins and transportation of these proteins into different cellular compartments, as described in more detail later.

Cell stress is here referred to as stresses that alter the homeostasis of the cell. These stresses includes those coming from increased temperature, ischemia, RONS, generation of abnormal proteins, uncoupling of oxidative phosphorylation or alterations in pH (Locke, 1997).

A hallmark for stressed cells is the increased expression of heat shock proteins. The roles of heat shock proteins upon cell stress are mainly linked to restoring cell homeostasis and prevent damage to proteins and alterations in cell function (Liu *et al.*, 2006; Mymrikov *et al.*, 2011). Additionally, they protect against future stress by binding to partly denatured proteins and other weakened structures (Lepore *et al.*, 2000; Koh, 2002; McArdle *et al.*, 2004*a*, 2004*b*). Their ability to prevent protein damage is mainly associated to their ability to prevent protein denaturation and aggregation, and help correct protein folding (Mymrikov *et al.*, 2011). When refolding is impossible, HSP can promote controlled proteasomal degradation of the damaged proteins (Mymrikov *et al.*, 2011). This is a biological important function, and a key feature for cell survival, as damaged proteins could affect normal cell function and be lethal for the cell.

Classes of heat shock proteins

Heat shock proteins are classified in groups according to their atomic mass in kilodalton, ranging from ~8 kDa (ubiquitin) to over 100 kDa (e.g. HSP110). A rough classification is usually to group them into large HSPs and small HSPs. The small heat shock proteins (sHSP) are usually referred to HSPs with an atomic mass less than 27 kDa, and the large HSPs are the ones with a mass over 27 kDa. Here I will shortly present some of the most commonly studied HSPs in human skeletal muscle in relation to training and exercise, and their biological role in cell homeostasis.

αB-crystallin

αB-crystallin is a 22 kDa protein that belongs to a family of α-crystallins. αB-crystallin was first identified in eye lenses and have important roles in protection against UV-damage and preserving transparancy of the lens (Andley, 2007). αB-crystallin is expressed in both heart and skeletal muscle cells, and is the HSP that appear in highest intracellular content in skeletal muscles (Larkins *et al.*, 2012*a*). The biological role is primarily linked to protein refolding, but αB-crystallin is also modulating gene expression (Mehlen *et al.*, 1996). In an unstressed/inactive state αB-crystallin are usually bound together and form large oligomers, but upon stress they dissociate and translocate to stressed or damaged areas (Ecroyd *et al.*, 2007). αB-crystallin have three phosphorylation sites, Ser^{19},Ser^{45} and Ser^{59} (Ito *et al.*, 1997). The exact role of these phosphorylation sites and how they are regulated is still not well understood, but we know that phosphorylation is important for the regulation of oligomer size (Ito *et al.*, 2001) and its chaperone activity (Ecroyd *et al.*, 2007; Peschek *et al.*, 2013), rather than its binding affinity to stressed proteins (Larkins *et al.*, 2012*b*). αB-crystallin has been shown to translocate to cytoskeletal structures after exercise, where it mainly binds in the Z-lines and other stressed or disrupted areas (Koh & Escobedo, 2004; Vissing *et al.*, 2009; Paulsen *et al.*, 2009; Jørgensen *et al.*, 2013).

Heat shock protein 27

HSP27 has quite similar structure as αB-crystallin, and forms large oligomers in unstressed cells which can form smaller sizes when phosphorylated (Ser^{15}, Ser^{78} and Ser^{82}) (Mymrikov *et al.*, 2011). Also, HSP27 have rather similar chaperone characteristics as αB-crystallin in stressed cells, and both translocates to cytoskeletal structures after exercise (Koh & Escobedo, 2004; Vissing *et al.*, 2009; Paulsen *et al.*, 2009). In contrast to αB-crystallin, which has been shown to mainly bind to desmin in the Z-lines, HSP27 binds to stressed actin microfilaments (Fischer *et al.*, 2002; Paulsen *et al.*, 2009). Thus, αB-crystallin and HSP27 has been identified to have important roles in stabilization and protection of cytoskeletal structures from exercise.

Heat shock protein 70

HSP70 consist of 13 isoforms, which all represent the HSP70 family (Kampinga *et al.*, 2009). In general they are divided in to three different groups;
A cognate isoform (heat shock cognate 73), which is constitutively expressed, and slightly inducible when exposed to cell stress, and in general have housekeeping roles. A glucose regulated protein (GRP75 or GRP78) that are induced by glucose deprivation and calcium influx. And an inducible form (HSP70 or HSP72) which is the most abundant form, and increase in content in response to stressful insults (Locke, 1997; Liu *et al.*, 2006).

When the cell is not exposed to stress, HSP70 have important functions to help the folding of newly synthesized proteins together with HSP60. HSP70 then transports the newly-folded proteins to the correct cellular compartment (Kiang & Tsokos, 1998; Stirling *et al.*, 2003). Together with HSP90, HSP70 is important for the final synthesis of certain hormone receptors and transcription factors (Young, 2001; Kampinga & Craig, 2010). Therefore HSP70 is considered a multifunctioning protein important for normal cell functions, and the cooperation with other HSPs makes a large repertoire of different functions.

In general, HSP70 have distinct roles compared to the small HSPs when exposed to stress. HSP70 has been linked to "general" recovery from damaging cell stresses, and are involved in remodeling of the damaged areas in the cell (Liu *et al.*, 2006). In agreement with this, HSP70 has been shown to increase in content in highly stressed cell compartments, e.g. the cytoskeleton, after damaging high-intensity exercise (Vissing *et al.*, 2009; Paulsen *et al.*, 2009).

Other HSPs

HSP60 and HSP90 are two other HSPs studied in relationship to exercise and training.
HSP60 is often considered to be a mitochondrial HSP, due to its main location and chaperone activity there. As mentioned above, HSP60 work together with HSP70 to fold newly synthesized proteins in the mitochondria. Additionally, HSP60 is central in helping protein transport across mitochondrial inner-membranes (Morton *et al.*, 2009*c*).
HSP90 is another important HSP where its main chaperone activity is related to folding and activation of several protein kinases, transcription factors and hormone receptors, thus, HSP90 plays a role in cellular signaling (Young, 2001; Kampinga & Craig, 2010). As pointed out earlier, the chaperone activity of HSP90 is dependent on the presence of HSP70, where their cooperation is important for HSP90s chaperone activity.

The role of heat shock proteins in exercise and training

Acute response to exercise

Since exercise induce a "mixture" of different stresses, exercised muscles display different HSP responses. The HSP response usually includes acute HSP translocation to weak areas, increased gene expression and a later increase in HSP protein levels. In general, the HSP response seen after exercise is dependent on exercise intensity and exercise form, where exercises inducing high-mechanical forces induce large responses (Koh & Escobedo, 2004; Paulsen *et al.*, 2007, 2009; Folkesson *et al.*, 2008). The acute response after such exercises usually involves rapid translocation of the small HSPs from the cytosol to bind to damaged cytoskeletal structures (Koh & Escobedo, 2004; Vissing *et al.*, 2009; Paulsen *et al.*, 2009; Jørgensen *et al.*, 2013). The translocation is followed by increased HSP expression the in the days after exercise (Thompson *et al.*, 2001, 2003; Paulsen *et al.*, 2007). This diplays the biological importance of the HSPs in recovery from damaging contractions, and their ability to "sense" damaged proteins.

The HSP response has also been observed after contractions considered less damging. Tupling et al. (2007) demonstrated increased HSP70 content in skeletal muscle the days after isometric contractions. Interestingly, the response was most evident in type 1 fibres, which most likely highlight higher stress put on the type 1 rather than type 2 fibres from the exercise performed in that study. It has also been shown increased HSP70 mRNA and protein levels after endurance exercise (Puntschart *et al.*, 1996; Febbraio & Koukoulas, 2000*a*; Walsh *et al.*, 2001; Khassaf *et al.*, 2001; Morton *et al.*, 2006). Interestingly, endurance exercise has not yet been found to increase the resting amount of the small HSPs (Morton *et al.*, 2006). However, endurance trained individuals have higher αB-crystallin and HSP60 protein levels, and display higher αB-crystallin/*CRYAB* gene expression, compared to untrained individuals (Yoshioka *et al.*, 2003; Morton *et al.*, 2008), indicating that endurance training may induce changes in αB-crystallin over a training period.

Since strength- and endurance exercise induce quite different adaptations and alterations in cell homeostasis, it is intriguing to understand what is the trigger for the HSP responses. As mentioned above, high intensity resistance exercise, with mostly eccentric contractions, induce large HSP repsonses. This form of exercise usually consists of high mechanical forces and may induce large myofibrillar disruptions (Clarkson & Hubal, 2002). This form of stress is usually termed *mechanical stress*. However, during muscle contractions other stresses also occur; increased intra muscular temperature, increased energy turnover and ischemic conditions to mention a few.

Increased temperature was the first stimuli discovered to induce an HSP response (Ritossa, 1962). In rat models, heating skeletal muscles to temperatures over 40°C have been shown to increase HSP content in the heated muscles (Oishi *et al.*, 2002) and to increase HSP binding to structures vulnerable to heat damage (Larkins *et al.*, 2012*b*). However, electrically stimulated muscles have been shown to increase HSP content, despite no increases in muscle temperature (McArdle *et al.*, 2001). Additionally, heating muscles to similar temperatures as observed during exercise (~39°C) does not increase muscle HSP content (Morton *et al.*, 2007). Taken together, this indicates that the HSP response after exercise is the result of exercise *per se* and not the increased temperature in the muscle and surrounding tissuses.

While the muscles contracts in increases energy turnover. This will induce a range of different stresses to the muscle like increased formation of RONS and ATP/glycogen depletion, both which has been shown to induce an HSP response.

RONS will be described in detail later, but shortly; RONS is reactive molecules that have the potential to induce oxidative damage to other molecules- often termed oxidative stress. Oxidative stress seem to activate the various HSPs differently; for instance HSP27 will undergo S-thiolation which reduces its oligomer size without phosphorylation and thus its activity (Mehlen *et al.*, 1995; Eaton *et al.*, 2002). HSP27 has also been suggested to reduce oxidative stress by controlling glutathione expression (Mehlen *et al.*, 1996), which act as an important antioxidant in human tissues especially skeletal muscle. Oxidative stress can also be "sensed" by HSF1 (Ahn & Thiele, 2003). This would result in the release of HSF1-bound HSP70 and increase its activity, additionaly increase expression of HSP70.

Ischemic stress occurs when the blood flow is restricted. In contracting muscles, blood flow is reduced with increased isometric force production, and contractions at low MVC[8] (15-20 % of MVC) is sufficient to alter muscle blood flow due to the increased intramuscular pressure (Sjøgaard *et al.*, 1988; de Ruiter *et al.*, 2007). When muscle cells (both heart- or skeletal muscles) are put under ischemic conditions, HSPs translocates seemingly to cytoskeletal structures. During ischemic conditions, the O_2 delivery to the (working) muscles is reduced, but it also increases ATP and glycogen depletion (Greenhaff *et al.*, 1993), which has been demonstrated to activate HSF1 and increase HSP70 content (Chang *et al.*, 2001; Febbraio *et al.*, 2002).

Chronic adaptations to training

Training induces systematic exposures of stressful insults to exercised muscles. Due to the responses described earlier it is conceivable that adaptations will occur in HSP content. List of studies reporting changes in HSP levels after a period of training is summarized in table 1.

Relatively few human studies have investigated the effects of training on HSP content. Gjøvaag & Dahl (2006) demonstrated an increase of 111 % in HSP70, and 71% increase in HSP27 after 5-8 weeks of strength training in previously untrained participants. This was also confirmed by Paulsen et al. (2011), where increased αB-crystallin, HSP27 and HP70 content was found in *m. vastus lateralis* after 11 weeks of strength training in muscles of previously untrained. Endurance training has also been shown to increase αB-crystallin and HSP60 levels in males, but not females (Morton *et al.*, 2009*b*) (sex-differences will be discussed later). In general, HSP levels seem to increase in response to training as a system to maintain cell homeostasis, and overcome the exercise stress put on the cells and prevent protein damage. However, as the muscle becomes more trained, and adapted to meet the current requirements, HSP levels stabilize. Even the typical HSP response seen after exercise is abolished in trained individuals, with no increases in mRNA or proteins after exercise, compared to muscles of untrained (Smolka *et al.*, 2000). Further increases can only be possible with more and higher stress levels put on the muscles. This was demonstrated in highly trained rowers, where increases in HSP70 only occurred after a period of very high intensity strength training, rather than high volume training- which usually consists of more lower intensity training (Liu *et al.*, 2000, 2004).

[8] Maximal voluntary isometric contraction

If the training sustain, or the intensity decreases, the HSP levels will also decrease (Liu *et al.*, 2004; Gjøvaag & Dahl, 2006). This illustrates how well this system is controlled to meet the current demands put on the muscle cells.

HSP70 has also been shown to be important in the protection against muscle atrophy. By overexpressing HSP70, muscle function was sustained by limiting atrophy in Duchenne muscular dystrophy disease[9] (Gehrig *et al.*, 2012). HSP70 seem also to be important in maintaining muscle function and increasing recovery from atrophy caused by disuse (Miyabara *et al.*, 2012). HSP70 seem to inhibit disuse atrophy through inhibition FOXO3[10] and NFκB (Senf *et al.*, 2008, 2010), both which regulate protein breakdown.

[9] Disorder caused by a mutation in the dystrophin gene which causes low muscle function and muscle atrophy
[10] Forkhead box O3 is a transcription factor that upregulates genes that are related to apoptosis and protein breakdown

Table 1. Human studies reporting changes in HSP levels after training.

Study	Subjects	Training	Duration	Findings
Gjøvaag & Dahl, 2006	Untrained males (*n*=12) and females (*n*=20)	Low to high intensity, low to high volume strength training. 3/week	5-8 weeks	Overall: ↑ HSP72, GRP75, HSP27. No differences between low or high intensity, or low or high volume
Gjøvaag *et al.*, 2006	Trained males (*n*=15)	Concentric or eccentric strength training 2-3/week	12 weeks	**Eccentric**: ↔ HSP72, GRP75. ↓ Ubiquitin. **Concentric**: ↔GRP75, Ubiquitin ↓HSP72
Liu *et al.*, 1999	Trained male athletes (*n*=10)	High to low intensity rowing	4 weeks	↑HSP70. Highest increase during high intensity training, before stabilizing with lower intensity, and decreasing with decreased intensity and training volume
Liu *et al.*, 2000	Trained male athletes (*n*=14)	High and low intensity strength training and rowing	24 days	↑HSP70. Highest increase during high intensity training compared to low intensity
Liu *et al.*, 2004	Trained male athletes (*n*=6)	High intensity strength training and low intensity rowing	6 weeks	↑HSP70 with high intensity strength training. ↔ Low intensity rowing
Morton *et al.*, 2009*a*	Active males (*n*=30)	High intensity endurance training 4/week	6 weeks	↑αB-crystallin, HSP70, HSP60 ↔ HSP27
Morton *et al.*, 2009*b*	Active males (*n*=5) and females (*n*=5)	Interval (high intensity) and continuous (moderate intensity) endurance training. 3/week	6 weeks	**Males**: ↑αB-crystallin after moderate intensity. ↑ αB-crystallin, HSP60 after high intensity. **Females**: ↔ αB-crystallin, HSP27, HSP60, HSP70 after both moderate and high intensity training
Paulsen *et al.*, 2012*a*	Untrained males (*n*=15)	Strength training; two groups exercising either 1 set lower body and 3 sets upper body, or 3 sets lower body and 1 set upper body. 3/week	11 weeks	Overall: ↑αB-crystallin, HSP27, HSP70. No differences between groups
Vogt *et al.*, 2001	Untrained males (*n*=30)	High or low intensity endurance training under normoxic or hypoxic conditions. 5/week	6 weeks	↑*HSPA4* mRNA after high intensity. ↔ *HSPA4* mRNA in low intensity. No differences between normoxic or hypoxic

Factors affecting the heat shock protein response and adaptation

In several studies, large individual differences in both the HSP response and training adaptation have been reported. These individual differences in HSP response might be related to initial high levels of HSPs, as trained individuals display hampered HSP response to exercise (Khassaf *et al.*, 2001; Morton *et al.*, 2006). Gjøvaag and Dahl (2006) reported a negative correlation between initial HSP levels and the increases after a training period. Paulsen et al. (2011) observed that strength training increased HSP content in *m. vastus lateralis*, but not in *m. trapezius*, which had higher levels of HSP before the training period. Overall, the HSP levels in the muscles, which usually come as a result of training, can dictate the magnitude of the HSP response and changes after a training period.

Morton et al. (2009) observed that females didn't increase αB-crystallin and HSP60 content compared to males in response to endurance training. However, Gjøvaag and Dahl (2006) did not observe any sex differences in levels of HSP27 or HSP70 after strength training. Further, male rats displayed higher HSP27, HSP70 and HSP90 content in slow twitch muscles compared to female rats, whereas HSP60 was only higher in fast twitch muscles of male rats (Voss *et al.*, 2003). One possible reason for the observed sex differences in HSP responses may be the circulatory sex hormone estrogen, which has been shown to inhibit NFκB signaling, and thereby inhibit stress signaling (Stice & Knowlton, 2008; Xing *et al.*, 2012). Additionally, estrogen has been shown to increase the phosphorylation of αB-crystallin and HSP27 in hearts of rats, which increased HSP activity (Hsu *et al.*, 2007). This indicates that females might have a higher HSP activity which might compensate for lower HSP content. Furthermore, castrated rats which lacked the male sex hormone testosterone, displayed blunted HSP70 response (mRNA and protein) in the heart after running (Milne *et al.*, 2006). Interestingly, rats that received testosterone supplementation had the same response as non-castrated rats. Based on these studies, we cannot exclude the possibility that sex differences can influence adaptations in HSP content in response to exercise.

Antioxidant defense systems

In addition to the heat shock proteins, muscle cells have developed a system of proteins to maintain cell homeostasis in response to free radicals (RONS). These endogenous antioxidant systems can be divided in to the enzymatic- and non-enzymatic antioxidants, with the goal to prevent oxidative damage.

Examples of the enzymatic antioxidants are the superoxide dismutases (SOD), catalase and glutathione peroxidases (GPx). Non-enzymatic antioxidants include glutathione, uric acid and co-enzyme Q_{10} to mention a few. In addition, humans ingest a wide range of dietary antioxidants, e.g. ascorbic acid/vitamin C, α-tocopherol/vitamin E, and phytochemicals – which can be considered non-enzymatic (Valko *et al.*, 2007; Peternelj & Coombes, 2011). In general, there is a close relationship between the enzymatic-, non-enzymatic and dietary antioxidants where they work together to neutralize RONS. The up- and downregulation of the enzymatic- and non-enzymatic antioxidants is mainly regulated by the levels of RONS/redox status in the cell (Powers & Lennon, 1999; Gomez-Cabrera *et al.*, 2006; Ji *et al.*, 2006).

Free radicals

Free radicals are molecules that have one or more unpaired electrons in one or more electron shells. Unpaired electrons give considerable reactive properties to molecules, which in return reacts with other molecules, usually referred to as secondary free radicals. At high levels, free radicals have potential to be harmful and give damaging effects to other molecules, e.g. proteins, nucleotides and lipids. Damage to other molecules can impair normal cell functions and ultimately cause necrosis (Peternelj & Coombes, 2011). The degree of oxidative damage is dependent on the balance between free radicals, antioxidants and other defense mechanisms (e.g. heat shock proteins).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are traditionally considered as bi-products of metabolism, together they have damaging properties. In general, ROS is produced in the mitochondria at theoretically eight different sites (Sena & Chandel, 2012), but Complex I and III in the electron transport chain is considered the main source (Powers *et al.*, 2011). ROS has also been shown to be produced at other sites outside the mitochondria, but is mainly driven by muscle contractions (McArdle *et al.*, 2001; Morales-Alamo & Calbet, 2014). These sites include NADPH oxidase in the sarcoplasmic reticulum and T-tubules, and xanthine oxidase in the cytosol. ROS is, however, also produced at rest, and considered a cause for muscle atrophy during a period of disuse (Powers *et al.*, 2012). Nitric oxide is considered the "main" RNS, and it is produced from L-arginine by NOS[11] (Dröge, 2002). Together with ROS they can form numerous different groups of free radicals, e.g. peroxynitrite, which is formed by singlet oxygen and nitric oxide (Powers & Jackson, 2008).

[11] Nitrogen oxide synthase

Antioxidants in human tissues

As mentioned above, human tissues contain a series of enzymatic and non-enzymatic antioxidants, and can use dietary antioxidants as defense against free radicals. Here I will briefly summarize the most important antioxidants, and how they are affected by exercise and training.

Enzymatic antioxidants

The enzymatic antioxidants use enzymatic reactions to reduce free radicals. In skeletal muscles the superoxide dismutases (SOD) and glutathione peroxidases (GPx) are the most commonly studied, and considered the main antioxidant enzymes in relation to training adaptation. These two enzymes are considered as endogenous antioxidants, meaning that they are produced *in vivo*.

The two isoforms of SOD found in skeletal muscles are CuZnSOD and mnSOD, which is encoded by the *SOD1* and *SOD2* gene, respectively. The two isoforms have different distribution within the muscle cell; CuZnSOD is mainly found in the cytosol, with some smaller fraction in the mitochondrial inner-membrane space, and mnSOD is mainly found in the mitochondrial matrix (Fukai & Ushio-Fukai, 2011). Due to the localization of mnSOD it has been suggested to be important for direct removal of ROS in the mitochondria, thus, important for normal mitochondrial function (Vincent *et al.*, 1999; Fukai & Ushio-Fukai, 2011). Both isoforms contain an active metal co-factor which is important for its antioxidant function by dismutation of $O_2^{\bullet}$- to oxygen and H_2O_2 (hydrogen peroxide; figure 2).

GPx has an important role as an antioxidant enzyme in cells. As SOD increases the formation of H_2O_2 in the cell, glutathione perodidase (GPx) reduce H_2O_2 to H_2O (figure 2). This occurs by the oxidation of GSH (reduced glutathione) to GSSG (oxidized glutathione).

Superoxide dismutases

As the name indicates, CuZnSOD contain copper and zinc, while mnSOD contains manganese as its cofactor. In general, both isoforms can be regulated by a wide range of stresses, including changes in RONS by exercise (Zelko *et al.*, 2002; Ji *et al.*, 2006). Cross sectional studies show that SOD activity and (mnSOD) content is higher in muscles and blood from trained, than untrained individuals (Jenkins *et al.*, 1984; Ohno *et al.*, 1992; Evelson *et al.*, 2002; Cazzola *et al.*, 2003; Morton *et al.*, 2008). This is a result of regular training, as training studies demonstrate that SOD activity and content increase after endurance- and strength training in both animals (table 2) and humans (table 3). In general, changes in enzyme activity are often a result of increased total enzyme content. Although, several studies have found increases in SOD activity and content, several studies also report no changes after a period of endurance training (table 2 and 3). However, several of these studies do not distinguish between the different isoforms of SOD which probably respond differently to training; For instance, some only find increases in the CuZnSOD isoform (table 2 and 3; e.g. Leeuwenburgh *et al.*, 1994, 1997; Parise *et al.*, 2005; García-López *et al.*, 2007), while others only in the mnSOD isoform (table 2 and 3; e.g. Higuchi *et al.*, 1985; Vincent *et al.*, 1999; Hollander *et al.*, 1999; Lambertucci *et al.*, 2007; Higashida *et al.*, 2011; Gliemann *et al.*, 2013). The increases in mnSOD after training seem to be higher in type 1 and type 2A fibers (high oxidative fibers), rather than type 2X fibers (Powers *et al.*, 1994*a*), and seem to increase more after high intensity than low intensity training (Powers *et al.*, 1994*a*; Scheffer *et al.*, 2012).

Glutathione peroxidases

Several isoforms of GPx exists, but the GPx1 isoform is the most abundant and studied in regard to training. Research is not consistent on whether GPx activity and content increase with training, however, most studies indicate that GPx will increase with training (table 2 and 3; e.g. Powers *et al.*, 1992; Criswell *et al.*, 1993; Leeuwenburgh *et al.*, 1994, 1997; Hollander *et al.*, 1999). However, some studies does not find any changes in response to training (table 2 and 3; e.g. Vincent *et al.*, 1999, 2000; Strobel *et al.*, 2011; Yfanti *et al.*, 2012). Likewise the SODs, GPx increase more after exercise with high intensity, than exercise with low intensity (Criswell *et al.*, 1993). Additionally, muscle fibers with high oxidative capacity (Type 1 and 2A), seem to gain the largest increases in response to endurance training (Powers *et al.*, 1994*a*), which probably is dictated by the activity level/recruitment pattern of the muscle fibers during endurance training.

Non-enzymatic antioxidants

As the name indicates, non-enzymatic antioxidants do not have catalytic antioxidant activities. However, they work directly as a substrate to reduce oxidized molecules by donating an electron. Several non-enzymatic antioxidants exist, but glutathione and uric acid is probably the two in most quantities and with importance in human skeletal muscles and plasma, respectively.

Glutathione

GPx is highly dependent on glutathione (GSH) to act as an antioxidant. Thus, GSH is considered one of the most important antioxidants in skeletal muscle cells (Wu *et al.*, 2004). Upon catalization of GSH by GPx, glutathione disulfide (GSSG) is formed, which is the oxidized form of GSH. GSSG can further be reduced back to GSH by glutathione reductase (GR) by the use of NADPH. GSH has also an important function to "regenerate" vitamin C and E back to an active antioxidant (Valko *et al.*, 2007). Since GSSG levels increase rapidly during submaximal exercise (Gohil *et al.*, 1988), it has been suggested to be a marker for oxidative stress. GSH (and GSSG) is found across most of human tissues, but the main site for synthesis is in the liver, where it is transported to different tissues through the bloodstream (Deneke & Fanburg, 1989; Lu, 1999). While some studies conclude that GSH can be increased with training in several different tissues, including muscle and plasma/blood (table 2 and 3; e.g. Kretzschmar *et al.*, 1991; Sen *et al.*, 1992; Marin *et al.*, 1993), others conclude that GSH is unchanged (table 3; Rabinovich *et al.*, 2001; Rodriguez *et al.*, 2012) and even decreased (table 2; Leeuwenburgh *et al.*, 1994) after training. This can probably be explained by the fact that muscles with mostly type 2 fibers usually display increases in GSH (Sen *et al.*, 1992; Leeuwenburgh *et al.*, 1997). However, as GSH can rapidly be oxidized to GSSG, most studies don't find an increase in only GSH content. It is, however, common to combine GSH and GSSG, since both are technically GSH, and report it as total GSH.

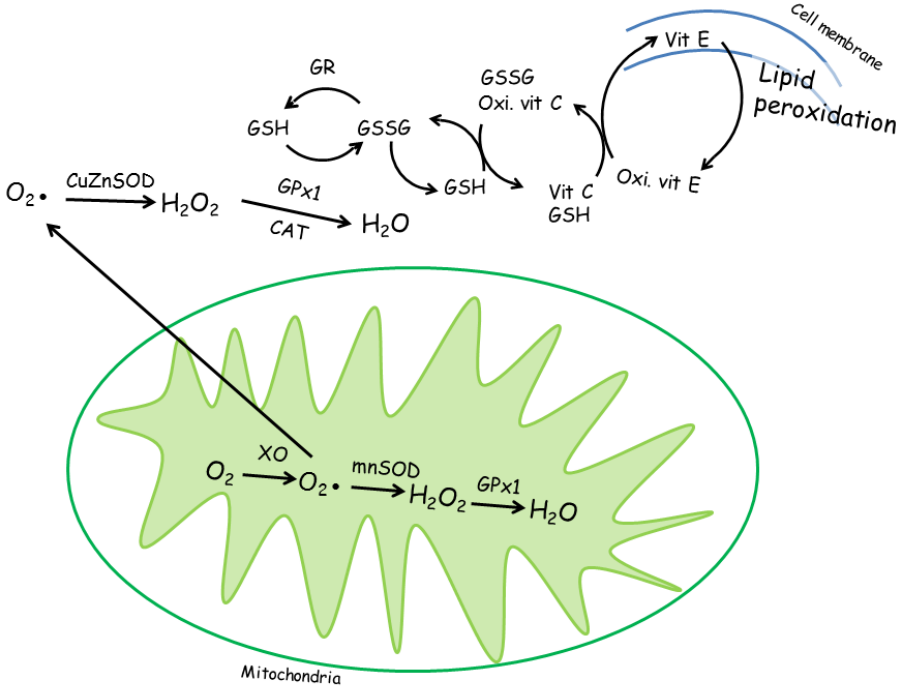

Figure 2. Simplified illustration over the redox regulation in the cell. Singlet oxygen is produced in the mitochondria or in the cytosol where it is reduced to H_2O in several steps involving different enzymes, where several antioxidant act together to eliminate RONS. Singlet oxygen can also leak from the mitochondria to the cytosol. Some antioxidant directly eliminate radicals, while other regenerate other antioxidants; e.g. lipid peroxidation is prevented by Vitamin E in the cell membrane. Vitamin E is further recharged by vitamin C, GSH or polyphenols (not illustrated here). O_2•, singlet oxygen; mnSOD, manangese superoxide dismutase; GPx1, glutathione peroxidase 1; CuZnSOD, copper-zinc superoxide dismutase, CAT, catalase; GSH, reduced glutathione; GSSG, oxidized glutathione; vit C, vitamin c/ascorbic acid; oxi. vit c, oxidized vitamin C/dehydroascorbic acid; vit E, vitamin E/tocopherol; oxi. vit E, oxidized vitamin E/alpha-tocopherylquinone.

Table 2. Animal studies reporting changes in endogenous antioxidant activity, mRNA and protein levels after endurance- or strength training.

Study	Tissue	Subjects	Training	Duration	Activity/mRNA/ Protein	Findings
Alessio & Goldfarb, 1988	Muscle (red and white gastroc)	Male rats (*n*=32)	Endurance training 5/week	18 weeks	Activity	↓ CAT ↔ SOD (total)
Criswell *et al.*, 1993	Muscle (gastroc, rectus femoris, soleus)	Female rats (*n*=36)	High intensity interval or moderate intensity continuous endurance training 5/week	12 weeks	Activity	↑ GPx in soleus after interval training ↑ SOD activity in soleus in both training groups
Gomez-Cabrera *et al.*, 2008	Muscle (soleus and gastroc)	Male rats (*n*=36)	Moderate intensity endurance training 5/week	3 weeks	mRNA	Results from non-antioxidant fed animals only: ↑ *SOD2, GPx*
Gore *et al.*, 1998	Muscle (DVL and SVL)	Female rats (*n*=21)	Endurance training 5/week	10 weeks	Activity, mRNA, protein	**Activity**: ↑ CuZnSOD and GPx (total) in DVL ↑ SOD (total) in both muscles ↔ mnSOD **mRNA**: ↑ *SOD1* in DVL ↔ *SOD2, GPx* **Protein**: ↑ CuZnSOD in SVL ↑ mnSOD in DVL
Higashida *et al.*, 2011	Muscle (epi and triceps)	Male rats (*n*=12)	Endurance training 6/week	3 weeks	Protein	↑ mnSOD ↔ CuZnSOD
Higuchi *et al.*, 1985	Muscle (DVL, SVL and soleus)	Female rats (*n*=17)	Endurance training 5/week	12 weeks	Activity	↑ SOD (total) in DVL and soleus, mnSOD in all muscles ↔ CAT, CuZnSOD in all muscles, SOD (total) in SVL

Table 2 continues

Hollander *et al.*, 1999	Muscle (DVL, SVL, plantaris, soleus)	Female rats (*n*=16)	Endurance training 5/week	10 weeks	Activity, mRNA and protein	**Activity**: ↑ CAT in DVL, GPx in DVL, mnSOD in DVL ↔ CAT, GPx and mnSOD in plantaris, soleus and SVL ↔ CuZnSOD in all muscles **mRNA**: ↔ *SOD2* in all muscles, *SOD1* in DVL, SVL and soleus ↓ *SOD1* in plantaris **Protein**: ↔ mnSOD, CuZnSOD in all muscles
Ji *et al.*, 1988	Muscle	Male rats (*n*=16)	Endurance training 5/week	10 weeks	Activity	↔ CAT, CuZnSOD, mnSOD ↓ GPx1/2
Lambertucci *et al.*, 2007	Muscle (soleus)	Male rats (young and old)	Low intensity endurance training 5/week	12 weeks	Activity and mRNA	**Young**: **Activity**: ↑ CAT, GPx, SOD (total), mnSOD ↔ CuZnSOD **mRNA**: ↑ *SOD2* ↔ *SOD1, GPx* **Old**: **Activity**: ↔ CAT, GPx, CuZnSOD ↓ SOD (total) mnSOD **mRNA**: ↓ *CAT, SOD2* ↔ *GPx, SOD1*

Table 2 continues

Laughlin *et al.*, 1990	Muscle (triceps brachii; medial head and long head; white and red portions, soleus and gastroc)	Male rats (*n*=78)	Endurance training 5/week	12 weeks	Activity	↔ SOD in all muscles, GPx gastrocnemius and soleus ↓ CAT in gastrocnemius and soleus
Leeuwenburgh *et al.*, 1994	Muscle (DVL, SVL, soleus)	Male rats (young, adult and old)	Moderate intensity endurance training 2-3/week	10 weeks	Activity and protein (GSH only)	**Activity**: ↑ GPx in young in vastus lateralis ↓ CAT in old **Protein**: ↓ GSH and tGSH in young in soleus
Leeuwenburgh et al., 1994	Muscle (DVL, SVL, soleus)	Male rats (young, adult and old)	Moderate intensity endurance training 2-3/week	10 weeks	Activity and protein (GSH only)	**Activity:** ↑ GPx in young in vastus lateralis ↓ CAT in old **Protein:** ↓ GSH and tGSH in young in soleus
Leeuwenburgh et al., 1997	Blood and muscle (DVL, soleus)	Female rats (n=21)	Endurance training 5/week	10 weeks	Activity and protein (GSH only)	**Activity:** ↑ GPx, CuZnSOD, SOD (total) in DVL ↔ mnSOD in all muscles **Protein:** ↑ GSH and tGSH in DVL ↓ GSH and tGSH in soleus
Marin et al., 1993	Blood and muscle (splenius, longissimus dorsi, triceps, extensor carpi radialis and gastroc)	Female dogs (n=22)	Endurance training 5/week	40 weeks	Protein	**Plasma:** ↑ tGSH **Muscle:** ↑ GSH in gastrocnemius

Table 2 continues

Nakao et al., 2000	Muscle (gastroc)	Male mice (n=12)	Endurance training 5/week	6 weeks	mRNA and protein	**mRNA:** ↔ *SOD1, SOD2* **Protein:** ↑ mnSOD ↔ CuZnSOD
Oh-ishi *et al.*, 1997	Muscle (soleus)	Male rats (*n*=38)	Endurance training 5/week	9 weeks	Activity, mRNA, protein	**Activity:** ↑ CuZnSOD, mnSOD, GPx ↔ CAT **mRNA:** ↔ CuZnSOD, mnSOD **Protein:** ↑ mnSOD ↔ CuZnSOD
Powers et al., 1992	Muscle (costal and crura diaphragm, intercostal muscle)	Female rats (young and old; n=40)	Endurance training 5/week	10 weeks	Activity	**Young:** ↑ GPx in costal diaphragm **Old:** ↔ GPx in all muscles

Table 2 continues

Powers et al., 1994	Muscle (red and white gastroc, soleus)	Female rats (n=72)	Low-, moderate- and high intensity endurance training with different durations: 30-60-90 min (total of 9 groups) 5/week	10 weeks	Activity	**Soleus:** ↑ SOD is a function of training duration up to 60 min ↔ CAT, GPx **Red gastrocnemius:** ↑ SOD in high intensity long duration, GPx as a function of training duration ↔ CAT **White gastrocnemius:** ↓ SOD for duration >60 min at all intensities ↔ CAT, GPx
Powers *et al.*, 1994*b*	Muscle (costal and crural region of diaphragm, plantaris, intercostal muscles)	Female rats (n=72)	Low-, moderate- and high intensity endurance training with different durations: 30-60-90 min (total of 9 groups) 5/week	10 weeks	Activity	**Costal diaphragm:** ↑ SOD (SOD greatest in 90 min), GPx at all intensities **Crural diaphragm:** ↑ SOD after ≥60 min duration ↔ GPx **Intercostal:** ↑ SOD after ≥60 min duration ↔ GPx **Plantaris:** ↑ SOD after ≥60 min duration, GPx

Table 2 continues

Ohkuwa et al., 1997	Blood and muscle (gastroc, soleus)	Female rats (young and old)	Endurance training 2/week	5 weeks	Protein	**Young:** ↑ GSH in plasma, gastrocnemius and soleus **Old:** ↔ GSH in plasma ↑ GSH in gastrocnemius ↓ GSH in soleus
Ryan et al., 2010	Muscle (dorsiflexors)	Male rats (young and old; n=28)	Strength training 3/week	4.5 weeks	Activity, mRNA and protein	Results from non-antioxidant fed animals only: **Young:** **Activity:** ↑ GPx, CuZnSOD ↔ CAT, mnSOD **mRNA:** ↔ *GPx, CAT, SOD1, SOD2* **Protein:** ↑mnSOD, CuZnSOD ↔ CAT, tGSH, GPx **Elderly:** **Activity:** ↑ GPx, CuZnSOD ↔ CAT, mnSOD **mRNA:** ↔ *GPx, CAT, SOD1, SOD2* **Protein:** ↑ tGSH ↔ CAT, CuZnSOD, mnSOD, GPx

Table 2 continues

Scheffer et al., 2012	Muscle (red brachioradialis)	Male rats (n=24)	Strength training: low, moderate, high intensity 4/week	12 weeks	Activity and protein	**Activity:** ↑ SOD (total), GPx (total) in moderate and high intensity groups ↓ CAT in moderate and high intensity groups **Protein:** ↑ CAT in all groups ↑ CuZnSOD, GPx1/2 in moderate and high intensity groups
Sen et al., 1992	Muscle (dogs: splenius, triceps, extensor carpi radialis and red gastroc. Rats: red gastroc, VL and longissimus dorsi)	Female dogs (n=22) and male rats (n=44)	Endurance training 5/week	55 weeks (dogs) 8 weeks (rats)	Activity and protein (GSH only)	**Dogs:** **Activity:** ↑ GPx in red gastrocnemius, extensor carpi radialis, triceps **Protein:** ↑ tGSH in red gastrocnemius **Rats:** **Activity:** ↑ GPx in red gastrocnemius, VL **Protein:** ↑ tGSH in red gastrocnemius, VL
Strobel et al., 2011	Muscle (red gastroc)	Male rats (n=48)	High intensity endurance training 4/week	14 weeks	Activity, mRNA and protein	Results from non-antioxidant fed animals only: **Activity:** ↔ GPx ↓ mnSOD **mRNA:** ↔ *SOD2, GPx* **Protein:** ↑ mnSOD

Table 2 continues

Quintanilha, 1984		Female rats (*n*=40)	Endurance training 5/week	10 weeks	Activity	↑ CAT, mnSOD, GPx
Venditti & Di Meo, 1996	Muscle (gastroc)	Male rats	Endurance training 5/week	10 weeks	Activity	↑ GPx
Venditti & Di Meo, 1997	Muscle (gastroc)	Male rats	Endurance training 5/week	10 weeks	Activity	↑ GPx
Venditti et al., 2014	Muscle (gastroc)	Male rats	Endurance training 5/week	10 weeks	Activity and protein (GSH only)	Results from non-antioxidant fed animals only: **Activity:** ↑ GPx **Protein:** ↔ GSH
Vincent et al., 1999	Muscle (costal diaphragm)	Female rats (n=15)	High intensity endurance training 4/week	12 weeks	Activity	↑ mnSOD ↔ CuZnSOD, GPx (total)
Vincent et al., 2000	Muscle (costal diaphragm)	Male rats (n=28)	Moderate intensity endurance training	5 days	Activity	↑ CAT, SOD (total) ↔ GPx1

VL, *m. vastus lateralis*; DVL, deep portion of *m. vastus lateralis* (high in type 2a fibers); SVL, superficial portion of *m. vastus lateralis* (high in type 2x fibers); Gastroc, gastrocnemius (red portion is usually high in type 2a fibers, white usually high in 2x fibers); Epi; *m.* epitrochlearis (high in type 2 fibers)*;* PBMC, peripheral blood mononuclear cell; GSH, reduced glutathione; tGSH, total glutathione (reduced glutathione+oxidized glutathione (GSSG); CAT, catalase; SOD, superoxide dismutase, mnSOD, manangese superoxide dismutase; CuZnSOD, copper-zinc superoxide dismutase; GPx, glutathione peroxidase; GPx1, glutathione peroxidase 1; GPx2, glutathione peroxidase 2; *SOD1*, CuZnSOD gene/mRNA; *SOD2*, mnSOD gene/mRNA.

Table 3. Human studies reporting changes in endogenous antioxidant activity, mRNA and protein levels after endurance- or strength training.

Study	**Tissue**	**Subjects**	**Training**	**Duration**	**Activity/mRNA/ Protein**	**Findings**
Aslan *et al.*, 1998	Blood (eryhrocytes)	Inactive males (*n*=15)	Endurance training 7/week	5 weeks	Activity	↔ SOD ↑ GPx
Devries *et al.*, 2008	Muscle (VL)	Obese and lean women (*n*=24)	Low-moderate intensity endurance training 2-3/week	12 weeks	Protein	**Obese**: ↓ CuZnSOD ↔ CAT, mnSOD **Lean**: ↔ CAT, CuZnSOD, mnSOD
Elosua *et al.*, 2003	Blood (eryhrocytes and whole blood)	Untrained males and females (*n*=17)	Moderate to high intensity endurance training 4-5/week	16 weeks	Activity	↑ GPx ↔ SOD
Fatouros *et al.*, 2004	Blood	Elderly males (*n*=19)	Low to moderate intensity endurance training 3/week	16 weeks	Activity	↓ GPx
García-López *et al.*, 2007	Blood (PBMC)	Active middle-aged males (*n*=32)	Strength or endurance training 2/week	21 weeks	Activity, mRNA and protein	**Activity**: ↔ CAT, GPx, mnSOD, CuZnSOD in both training groups **mRNA**: **Strength**: ↑ *CAT, SOD1, SOD2, GPx1* **Endurance**: ↑ *Gpx1, SOD2* ↔ *CAT, SOD1* **Protein**: ↑ CuZnSOD (strength only) ↔ CAT, GPx, mnSOD (CuZnSOD in endurance only)

Table 3 continues

Gliemann *et al.*, 2013	Muscle (VL)	Inactive elderly males (*n*=27)	High intensity interval/endurance training and whole body strength training ("Crossfit") 3/week	8 weeks	Protein	↓GPx ↔ CAT, CuZnSOD ↑ mnSOD
Hellsten *et al.*, 1996	Muscle	Men (*n*=11)	High intensity sprint interval/endurance training 3/week	6 weeks	Activity	↔ GPx, SOD
Linke *et al.*, 2005	Muscle (VL)	CHF patients and healthy males (*n*=35)	Moderate intensity endurance training 4-6/week	26 weeks	Activity, mRNA and protein	**Activity**: ↑ CAT, GPx (both; CHF patients only) ↔ SOD (total) **mRNA**: ↔ *CAT, SOD1, SOD2, GPx* **Protein**: ↔ CAT, CuZnSOD, mnSOD
Miyazaki *et al.*, 2001	Blood (eryhrocytes)	Untrained males (*n*=9)	Moderate intensity endurance training 5/week	12 weeks	Activity	↑ GPx (total), SOD (total) ↔ CAT
Ohno *et al.*, 1988	Blood (erythrocyte)	Untrained males (*n*=7)	Endurance training 6/week	10 weeks	Activity and protein	**Activity**: ↑CAT ↔ CuZnSOD **Protein**: ↑CAT ↔ GSH, GPx, CuZnSOD
Parise *et al.*, 2005	Muscle (VL)	Untrained elderly males (*n*=12)	Strength training 3/week	12 weeks	Activity	↑CAT, CuZnSOD ↔ SOD (total), mnSOD

Table 3 continues

Rabinovich *et al.*, 2001	Muscle (VL)	Elderly COPD patients and healthy males (*n*=22)	High intensity interval/endurance training 5/week	8 weeks	Protein	**COPD**: ↔ GSH and tGSH **Healthy**: ↑ GSH, tGSH
Rabinovich *et al.*, 2006	Muscle (VL)	Elderly COPD patients and healthy males (*n*=25)	High intensity interval/endurance training	8 weeks	Protein	**COPD**: ↑ GSH (low BMI patiens) ↔ GSH (normal BMI patiens) **Healthy**: ↑ GSH
Ristow *et al.*, 2009	Muscle	Untrained and pre-trained males (*n*=40)	Moderate intensity endurance training and moderate intensity strength training 5/week	4 weeks	mRNA	Results from non-antioxidant fed participants only: ↑ *SOD1, SOD2, GPx1* (both untrained and pre-trained)
Rodriguez *et al.*, 2012	Muscle (VL) and blood	Elderly COPD patients and healthy males and females (*n*=30)	High intensity interval/endurance training 5/week	8 weeks	Activity and protein	**COPD**: **Activity**: ↔ CAT, SOD (total) **Content**: ↔ plasma and muscle GSH, CAT, mnSOD **Healthy**: **Activity**: ↔ CAT, SOD (total) **Protein**: ↑ mnSOD ↔ plasma and muscle GSH, CAT

Table 3 continues

Tessier, 1995	Muscle (VL)	Active males (*n*=24)	Endurance training 3/week	10 weeks	Activity	↔ GPx
Tiidus *et al.*, 1996	Muscle (VL)	Untrained males and females (*n*=13)	Moderate intensity endurance training 3/week	8 weeks	Activity	↔ CAT, SOD (total), GPx
Tonkonogi *et al.*, 2000	Muscle (VL)	Untrained males and females (*n*=8)	Moderate to high intensity endurance training 4/week	6 weeks	Activity Protein	**Activity**: ↔ CuZnSOD, mnSOD, GPx **Protein**: ↔ tGSH
Yfanti *et al.*, 2011*a*	Muscle (VL)	Active males (*n*=21)	High to moderate endurance training 5/week	12 weeks	Protein	↑ mnSOD
Yfanti *et al.*, 2012*b*	Muscle (VL)	Active males (*n*=21)	High to moderate endurance training 5/week	12 weeks	mRNA and Protein	**mRNA**: ↔ *CAT, SOD1, GPx1* **Protein**: ↔ CAT, CuZnSOD, GPx1
Zago *et al.*, 2010	Blood	Untrained elderly males with pre-hyperten-tion (*n*=118)	Low to moderate intensity endurance training 3/week	26 weeks	Activity and mRNA	↔ SOD (total)

VL, *m. vastus lateralis*; PBMC, peripheral blood mononuclear cell; GSH, reduced glutathione; tGSH, total glutathione (reduced glutathione+oxidized glutathione (GSSG); CAT, catalase; SOD, superoxide dismutase, mnSOD, manangese superoxide dismutase; CuZnSOD, copper-zinc superoxide dismutase; GPx, glutathione peroxidase; GPx1, glutathione peroxidase 1; GPx2, glutathione peroxidase 2; SOD1, CuZnSOD gene/mRNA; SOD2, mnSOD gene/mRNA.

Uric acid

Uric acid is present in literally all tissues, but highest levels are found in plasma, where most are in the form of urate (Glantzounis *et al.*, 2005). Uric acid is considered an important antioxidant because of its great potential to neutralize several types of RONS (Ames *et al.*, 1981; Glantzounis *et al.*, 2005). Uric acid is actually a bi-product of purine metabolism and thus uric acid levels is highly dependent on the diet; where a diet rich in purines, e.g. red meat, increase (Choi *et al.*, 2004), while diets rich in antioxidants/vitamin C can decrease uric acid levels (Juraschek *et al.*, 2011). Since uric acid levels are associated with the diet, studies investigating the effects of training on uric acid are limited.

Uric acid has been shown to increase in plasma during high intensity exercise (Green & Fraser, 1988; Mastaloudis *et al.*, 2001), which can indicate increased production or release to the blood stream. Further, this increase in uric acid seem to be important for maintenance of muscle uric acid levels, as both uric acid usage and uptake increase in active muscles (Hellsten *et al.*, 1997, 1998). Thus, it is plausible that uric acid levels or production might be affected by training.

Despite being an important antioxidant in humans, high levels of uric acid in plasma has been linked to several diseases like gout, kidney disease, diabetes, cardiovascular disease and hypertension (Choi *et al.*, 2005; Feig *et al.*, 2008; Chaudhary *et al.*, 2013). Additionally, uric acid levels has been associated with increased levels of circulatory inflammatory markers (Ruggiero *et al.*, 2006; Lyngdoh *et al.*, 2011).

Dietary antioxidants

The dietary antioxidants are molecules that the body gets from the diet which has antioxidant properties, and can include several thousand different compounds. Here I will briefly go through the antioxidants used in the studies included in this PhD thesis.

Vitamin C

Ascorbic acid, vitamin C, is a water-soluble vitamin with antioxidant properties (for more details about vitamin C see Carr & Frei, 1999). Most mammals can produce vitamin C from glucose, but in humans (and guinea pigs) a mutation in the gene coding for L-gulonolactone oxidase, which synthesize vitamin C, makes the enzyme not functional. This makes vitamin C an essential nutrient for humans, and deficiency could lead to the fatal disease scurvy. Vitamin C is obtained mainly from fruits and vegetables like oranges, kiwi fruit, potatoes and broccoli, but can also come from synthetic sources. As an antioxidant, vitamin C can directly act as a RONS scavenger, but has also the ability to regenerate the antioxidant vitamin E from its oxidized form (Niki, 1991), as described under.

Vitamin E

Tocopherol, or vitamin E, is a group of fat-soluble vitamins with antioxidant properties, mainly to protect against lipid peroxidation, hence it is located in the cell membrane (Brigelius-Flohe & Traber, 1999). Vitamin E deficiency can lead to severe abnormalities in the neuromuscular system and myopathies (Brigelius-Flohe & Traber, 1999). Vitamin E is usually found in foods high in fat like safflower oil and almonds.

When vitamin E acts as an antioxidant, it becomes a radical and gets pro-oxidative properties. However, vitamin C and GSH has the ability to "regenerate" vitamin E so it becomes an active antioxidant again. The process involving vitamin C will again form the vitamin C radical which needs to be further reduced by GSH (figure 2; Valko *et al.*, 2007*b*). This process is important for redox homeostasis and control in human tissues. It has also been suggested that polyphenols, which consists of several thousand compounds, have the ability to recharge these antioxidants, e.g. catechins, a flavonoid found in tea, has been shown to recharge vitamin E in the cell membrane (Aldini *et al.*, 2003).

RONS and adaptations to training and exercise

The traditional view of RONS is that it's dangerous and harmful to the cells. This view has, however, changed drastically in the recent years. There is now strong evidence that RONS can also act as important signaling molecules or act as secondary messengers (Gomez-Cabrera *et al.*, 2008; Powers *et al.*, 2010). However, the important signaling function of RONS can only occur at moderate concentrations, where short periods of RONS exposure can activate signaling pathways, whereas high levels, especially over long periods of time, may result in increased oxidative stress, which can impair cell function or promote cell death (Ji *et al.*, 2006).

There are many redox sensitive signaling pathways in skeletal muscles, but three pathways stand out as important for several exercise induced adaptations; MAPKs[12], PGC1α[13] and NFκB. Alterations in these, by e.g. antioxidant supplementation, can in theory affect adaptations to training. However, this is a highly debated topic (Holloszy *et al.*, 2012; Gomez-Cabrera *et al.*, 2012), and still under heavy research.

Alterations in exercise induced adaptations by antioxidants

The effects of RONS on training adaptations, and the possible interfering effects of antioxidant supplementation on these adaptations has been under intensive research the last decade, and summarized in several reviews (e.g. Peternelj & Coombes, 2011; Nikolaidis *et al.*, 2012).

One of the first studies to show a possible interference by vitamin C on training adaptations was a study conducted on greyhounds (Marshall *et al.*, 2002). Here, in a cross over design, the dogs seemed to have increased racing time, i.e. reduced running speed, when supplemented with high doses of vitamin C.

Gomez-Cabrera et al. (2005) showed that, by supplementing rats with allopurinol, the exercise induced increases in RONS was blunted. This resulted in blunted activation of the MAPKs p38 and ERK1/2, and reduced DNA binding of NFκB, thus reduced upregulation of the antioxidant enzyme mnSOD (*SOD2*). A few years later, the same group demonstrated that vitamin C supplemented rats had blunted *SOD2* and *GPx1* gene expression, additionally it also inhibited the exercise induced increases in mitochondrial biogenesis, measured by blunted PGC1α and cytochrome C protein content (Gomez-Cabrera *et al.*, 2008). This effect was confirmed in humans by Ristow et al. (2009) showing blunting effects of vitamin C and E on several genes related to training adaptations (e.g. *PGC1α*, *SOD2*, *GPx1*). The two last studies presented here has, however, been under heavy criticism (Higashida *et al.*, 2011; Holloszy *et al.*, 2012), especially since they are mainly based on resting mRNA levels, rather than changes in protein content. However, recently Paulsen et al. (2014) confirmed that vitamin C and E supplementation might blunt mitochondrial biogenesis, indicated by blocking of training induced increases in muscle COX4[14] protein content.

[12] Mitogen-activated protein kinases. Family of kinases that play an essential role in the regulation of gene expression and metabolism, where ERK1/2, p38 MAPK and JNK can be affected by ROS (Kramer & Goodyear, 2007)

[13] Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, co-activator and key regulator of mitochondrial biogenesis

[14] Cytochrome C oxidase subunit 4; mitochondrial ennzyme in the respiratory chain. COX4 can be used as a marker for mitochondria content (Larsen *et al.*, 2012)

It has also been demonstrated that vitamin C supplementation attenuates overload induced hypertrophy in rats, likely through blunting of p70S6K[15] and ERK1/2 activation (Makanae *et al.*, 2013). From the studies presented here, it is likely that antioxidant supplementation at high dosages may have negative effects on training adaptations. However, several research groups have failed to reproduce these studies. For instance in a series of papers Yfanti et al. (2010, 2011, 2012) did not observe any negative effects of vitamin C and E supplementation on training adaptations in well-trained males, including performance variables (VO_{2max}, power output), markers of mitochondrial biogenesis and antioxidant systems. Similarly, no negative effects on muscle adaptations was reported after training in elderly males supplemented with resveratrol (Gliemann *et al.*, 2013). Additionally, Higashida et al. (2011) did not report any negative effects of vitamin C and E supplementation on any training adaptations in rats.

Due to the effects of RONS on HSF1 activation (Ahn & Thiele, 2003) (the main transcription factor for HSP synthesis), the effects of antioxidant supplementation on HSPs has been investigated by several researchers, and mainly in the response to endurance exercises.

Khassaf et al. (2003) investigated the effects of vitamin C supplementation on skeletal muscle and lymphocyte HSP60 and HSP70 response (increases in HSP days after exercise). The results showed that vitamin C supplementation was able to increase HSP70 content after 8 weeks, thus blunt the HSP response at 2 days after exercise. For HSP60, neither increases in protein content nor an HSP60 response was seen after exercise. Fascinating, vitamin C supplementation seemed to abolish increases in HSP60 and HSP70 content when lymphocytes were exposed to RONS. Similar results was reported by Jackson et al., (2004) when supplementing with vitamin E. Later studies indicate that vitamin C and E may be able to blunt the HSP response at a transcriptional level (Fischer *et al.*, 2006). Lower *HSPA4* mRNA (HSP70) has been seen after infusion of the drug N-acetylcysteine, which has powerful antioxidant properties, during cycling to fatigue (Petersen *et al.*, 2012).

Overall, research concerning the effects of antioxidant supplementation on training adaptations is inconclusive and does not give any clear answers whether it may or may not have negative (or positive) effects on training adaptations. However, high dosages of antioxidant supplementation may negatively affect the acute HSP response to exercise, and probably also the following training adaptations in this system over prolonged periods of training.

[15] Serine/threonine kinase downstream of mTOR (mammalian target of rapamycin) important for regulation of protein synthesis after exercise

Summary

Muscular training adaptations do not only involve the normally investigated increases in muscle size (strength training) or mitochondrial capacity (endurance training), because several homeostatic systems need to follow. As discussed above, these systems react to different types of exercise-induced stress. For instance, the HSPs can be induced by different types of stress which can be prompted by different types of exercise. Several HSPs increase in content after periods of both endurance and strength training, but changes in HSP levels seem to be more pronounced after exercise with high intensity – which logically induces higher stress than low intensity. Correspondingly, the acute responses after a single bout of exercise are also affected by intensity, where higher intensity induces larger increases in protein content. Some studies have investigated the translocation of the HSP after bouts of high-force eccentric exercise. These studies show that the HSPs rapidly translocate to structures that are highly stressed after this form of exercise which mostly includes proteins in cytoskeletal structures. Translocation of the HSP after lower intensity exercise is, however, still unknown. Importantly, ischemia *per se* can result in similar translocation to cytoskeletal structures as observed after high-force exercise. It is therefore likely that HSPs also translocate to these structures after exercises not associated with large grade of muscle damage, e.g. "normal" strength training or low load occlusion training (low load blood flow restricted strength exercise).

The formation of RONS during exercise, can, together with the other types of stress from exercise, induce damage to proteins, nucleotides and lipids, and cause improper cell functions. The neutralization of the damaging properties of these reactive species is caused by the interaction of a network of several antioxidants. Most of these antioxidants are produced in the cell (endogenous antioxidants), but several important antioxidants must come from the diet (exogenous antioxidants). The endogenous antioxidants seem to increase as an adaptation to both endurance and strength training, and exercise intensity and duration – which most likely reflects levels of oxidative stress in the muscle cell. To help fight oxidative stress and reduce high concentration of RONS, many athletes take antioxidant supplements (Sobal & Marquart, 1994). As this might seem logical and beneficial when doing hard and frequent training, it might interfere with important cellular processes related to training adaptations. Several studies have reported negative effects of high doses of antioxidants on training adaptations like in the HSPs and the endogenous antioxidants. However, conflicting results exists, which might come from the studied population, tissues examined, doses and timing of the antioxidant supplements.

Research aims

This PhD thesis consists of four research projects which had the overall objective to investigate the stress responses and training adaptations in heat shock proteins and antioxidant systems in human skeletal muscles (*m. vastus lateralis*) after exposure to different exercise stresses.

The projects were divided into two categories:

1. Investigate the "acute" (single session) HSP response and long-term training adaptations in HSPs and antioxidant systems in skeletal muscles to low load blood flow restricted exercise (BFRE) (paper 1 and 2).
2. Investigate the effects of high doses of vitamin C and E (antioxidants) supplementation combined with high intensity endurance or strength training on adaptations in HSPs and antioxidant systems in skeletal muscles (paper 3 and 4).

The hypotheses concerning category 1 were:

1. Low load blood flow restricted exercise (BFRE) will cause a larger acute HSP (HSP27, αB-crystallin and HSP70) translocation to cytoskeletal structures than work-matched free flow exercise.
2. Low load BFRE will cause the same acute HSP (HSP27, αB-crystallin and HSP70) translocation to cytoskeletal structures as heavy load strength training.
3. The acute HSP response with low load BFRE will be larger in type 1 than in type 2 fibers.
4. Low load BFRE and heavy load strength training will induce an upregulation in HSP and antioxidant gene expression.
5. The acute HSP response after exercise will be reduced after 12 weeks of BFRE and heavy load strength training.
6. 12 weeks of BFRE and heavy load strength training will increase HSPs (HSP27, αB-crystallin and HSP70) content in skeletal muscle.
7. 12 weeks BFRE and heavy load strength training will increase endogenous antioxidant (mnSOD and GPx1) content in skeletal muscle.

The hypotheses concerning category 2 were:

1. Vitamin C and E supplementation will blunt increases in HSP and antioxidant gene expression by blunting NFκB activation acutely after a single session of high intensity interval running exercise.
2. Vitamin C and E supplementation will reduce the training induced increases in muscle HSP content (HSP27, αB-crystallin and HSP70) after 11 weeks of high intensity endurance training.
3. Vitamin C and E supplementation will reduce the training induced increases in endogenous antioxidant content (GSH, mnSOD and GPx1) in skeletal muscle after 11 weeks of high intensity endurance training.

4. Vitamin C and E supplementation will blunt increases in muscle HSP (*HSPB1, CRYAB* and *HSPA4*) and antioxidant (*SOD2* and *GPx1*) mRNA expression by reduced NFκB activation after a single session of heavy load strength training.
5. Vitamin C and E supplementation will reduce the training induced increases in muscle HSP content (HSP27, αB-crystallin and HSP70) after 10 weeks of heavy load strength training.
6. Vitamin C and E supplementation will reduce the training induced increases in muscle antioxidant content (GSH, mnSOD and GPx1) after 10 weeks of heavy load strength training.

Methods

The PhD thesis presents data from four different studies:

1. Acute HSP response in skeletal muscle after low load blood flow restricted resistance exercise (paper 1).
2. Acute and long term effects of low load blood flow restricted resistance exercise on HSPs and antioxidant systems in skeletal muscles of previously untrained females (paper 2).
3. Effects of vitamin C and E supplementation on antioxidant systems and HSPs in response to high intensity endurance training in trained males and females (paper 3).
4. Effects of vitamin C and E supplementation on antioxidant systems and HSPs in response to high intensity strength training in trained males and females (paper 4).

Participants

Paper 1 included seven males and two females (*n*=9; age 26±3 years, height 177±7 cm, body weight 79±11 kg). All participants were physically active with varying degree of strength training experience.

Paper 2 included nine untrained females (age 22±1 years, height 169±3 cm, body weight 69±5 kg).

Paper 3 included 19 male and 18 females (*n*=37; age 23±4 years, height 176±10 cm, body weight 71±13 kg). All participants were physical active, conducting regularly endurance training (1-4 times per week) before starting the study.

Paper 4 included 18 male and ten females (*n*=28; age 25±5 years, height 175±8 cm, body weight 74±13 kg. All participants were physical active, conducting regularly strength training (1-4 times per week) before start of the study.
All participants in all studies gave written informed consent before entering the study, and were informed about potential risks related to the experiment. The studies were approved by the Regional Ethics Committee of Southern Norway and were performed in accordance with the Helsinki Declaration.

Experimental design

Paper 1

One week before the experiment, one repetition maximum (1RM) of unilateral knee extension and a familiarization session was tested in a knee extension machine (Gym2000, Vikersund, Norway). The main experiment was carried out with partial blood-flow restriction induced by a 13.5-cm-wide pressure cuff connected to a tourniquet system (Zimmer A.T.S 2000, Zimmer Patient Care, Dover, OH, USA). The cuff was wrapped around the proximal part of the thigh and inflated to 90 or 100 mmHg, for females and males, respectively. Following a light 5-min warm-up on a stationary bicycle (without BFR), five sets to failure at 30% of 1RM was performed during BFR on one randomly selected leg (dominant/non-dominant). Subsequently, with the same relative load, the corresponding number of repetitions was performed with the other leg without BFR. Forty-five seconds of rest was given between sets.

Before and 1, 24 and 48 hours after exercise isometric maximal voluntary contractions (MVC; 90° knee angle) were measured for the knee extensor in both legs. These results was published in Wernbom et al. (2012), and will be included in correlation analyses in the present paper.

Under local anesthesia (Xylocain adrenalin, 10 mg/mL+5 mg/mL; AstraZeneca, London, UK) approx. 200 mg (2–3 x 50–150 mg) of muscle sample was obtained using a modified Bergström technique. Muscle biopsies were sampled from the mid-portion of *m. vastus lateralis* before, 1, 24 and 48 h after exercise.

Paper 2

Participants performed 12 weeks training of unilateral knee-extension (Technogym, Gambettola, Italy) twice per week. In a randomized order, one leg was chosen to exercise with partial blood flow restricted (BFRE) or high load resistance exercise (HLS). The BFRE consisted of 5 sets to failure at 30% of 1 RM with 45 seconds rest between sets. The 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 intensity (10 RM) and the second day was performed with high intensity (6 RM) training.

The partial blood flow restriction was induced by a 18 cm wide pressure cuff (Delfi Medical, Vancouver, Canada) connected to a tourniquet system (Welch Allyn, NY, USA). The cuff was wrapped around the proximal part of the thigh and inflated to 90 mmHg the first 6 weeks of training. The cuff was then inflated to 100 mmHg the last 6 weeks of training.

Unilateral knee-extension 1RM for each leg was determined before and after the training period using the same unilateral knee-extension apparatus used during the training.

Muscle biopsies were sampled from *m. vastus lateralis* before and 1 hour after the first and last exercise using the same procedure as described under study 1.

Paper 3

Supplements

The C and E vitamin and placebo pills were produced under Good Manufacturing Practice (GMP) requirements at Petefa AB (Västra Frölunda, Sweden). Each vitamin pill contained 250 mg of ascorbic acid and 58.5 mg DL-alpha-tocopherol acetate. The placebo pills had the same shape and appearance as the vitamins pills. All supplements were stored in unlabeled boxes.

The participants ingested two pills (500 mg of vitamin C and 117 mg vitamin E) 1-3 hours before every training session and two pills in the hour after training. On non-training days the participants ingested two pills in the morning and two pills in the evening. The intake of pills was confirmed with an online training diary. Thus, daily dosage was 1000 mg of vitamin C and 235 mg vitamin E. The total supplemental dosage of vitamin C was ~13 times higher than the recommended daily dietary allowance in the Nordic countries, and ~23 times higher for vitamin E.

Beside the supplementation given in the study, the participants were asked not to take any form of nutritional supplement. They were also asked to not drink more than two glasses of juice and four cups of coffee or tea per day. Juices especially rich in antioxidants, such as grape juice, were to be avoided.

Training

The training consisted of three blocks over 11 weeks, where three running sessions were conducted the three first weeks. Thereafter the participants increased to four sessions per week. The exercise durations increased linearly in each exercise block. The training sessions were a mix of low- (60 min), moderate- (30 min) and high intensity (intervals 4-6x 4-6 min). Exercise intensity was calculated from maximal heart rate and the participants' perceived exertion using the Borg Scale (RPE). During each training session heart rate/exercise intensity were monitored and logged using a hearth frequency monitor (RS800CX, Polar Electro Oy, Tempere, Finland), and type of exercise and session RPE were logged using an online training log after each exercise. For variation and motivation, participants were allowed to do alternative exercise forms (e.g. cycling, cross country skiing) once per week, including a maximum of two bouts of other activities in addition to the planned training sessions. Muscle biopsies from *m. vastus lateralis* were collected before and after the training intervention using the same procedure as described under study 1.

Acute exercise bout

After five weeks of training, a subsample of participants (*n*=16) volunteered to complete an exercise bout of 4x 4 min high intensity interval training (>90 % of maximal heart rate [HR_{max}]). The exercise session was performed on a treadmill (ELG 90/200 Sport, Woodway GmbH, Weil am Rhein, Germany). Exercise intensity was monitored using a heart rate monitor. VO_2 was measured during the last 1.5 min of each 4 min interval using a mixing chamber (Oxycon Pro, Erich Jaeger GmbH, Hoechberg, Germany), and blood lactate (La^-) was measured (YSI 150 Sport Lactate Analyzer, YSI Inc., Yellow Springs, OH, USA) immediately after each interval using a finger stick. The participants ingested the supplements one to three hours before and immediately after the exercise bout. Blood samples were collected before exercise and 10, 90 and 180 min post exercise. Muscle biopsies were collected pre exercise (and supplementation) and 90 min post exercise using the same procedure as described under study 1.

Paper 4

Training

After a baseline maximal strength test (1RM) the participants were randomly assigned to receive vitamin C and E or a placebo supplements. The same supplements under the same procedures as described under paper 3 were done in this experiment.

The exercise consisted strength training with heavy loads (6-11RM) for 10 weeks. Three to four sets per exercise was separated by short rest periods (1-1.5 min). The exercise program included exercises for all major muscle groups in a 4-split exercise program (two upper- and two lower body sessions per week), with the goal to stimulate both maximal strength and muscle growth. The exercise and control of supplementation was monitored and logged using an online training diary. For variation and motivation participants were allowed to do alternative exercise forms (e.g. cycling, cross country skiing) once per week, including a maximum of two bouts of other activities in addition to the planned training sessions.

Acute exercise session

After 4-6 weeks, 15 participants volunteered to do an acute exercise experiment. The exercise session included 4x 10RM of leg press and knee-extension, with 1 min rest between sets and 3 min rest between exercises. Muscle biopsies were collected from *m. vastus lateralis* before and 1.5 and 2.5 hours after the exercise bout, as described later.

Participants ingested the supplements, vitamin C and E or placebo, together with a standardized breakfast (3 g oat per kg body weight boiled in water with 5 g sugar) two hours before meeting in the laboratory. A new dose of supplements was taken after the exercise bout.

Analyses

Homeogenization and protein quantification

Muscle tissue was homogenized using a commercial homogenization buffer (Cat#78510, T-PER/Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA) and protease and phosphatase inhibitors (Cat#1861281, Halt protein and phosphatase inhibitor cocktail, Thermo Scientific) and EDTA (Cat#1861274, Thermo Scientific).

Some samples were also homogenized and fractionated into cytosol-, membrane-, nuclear- and cytoskeletal fractions, using a commercial fractionation kit (ProteoExtract Subcellular Proteome Extraction Kit, Cat#539790, Calbiochem, EMD Biosciences, Schwalbach, Germany).

Protein concentrations were measured with a commercial kit (BioRad DC protein micro plate assay, Cat#0113, Cat#0114, Cat#0115, Bio-Rad, Hercules, CA, USA), and quantified using a filter photometer (Expert 96, ASYS Hitech, Cambridge, UK) and the provided software (Kim, ver. 5.45.0.1, Daniel Kittrich).

Protein immunoblot

Proteins extracted from the muscle samples were analyzed by the western blotting technique. Denatured proteins were separated in 4-12% SDS-PAGE gels in cold MES running buffer (NuPAGE MES SDS running buffer, Invitrogen, Life technologies). Proteins were thereafter transferred onto PVDF-membranes (Cat#162-0177, Bio-Rad or iBlot Gel transfer stacks, Cat#IB4010, Invitrogen). Membranes were blocked at room temperature for 2 hours or overnight at 4°C in a 5% fat free skimmed milk and 0.05% TBS-t solution (TBS, Cat#170-6435, Bio-Rad; Tween 20, Cat#437082Q, VWR International, Radnor, PA, USA; Skim milk, Cat#1.15363, Merck, Darmstadt, Germany).

Blocked membranes were incubated in antibodies against the desired protein (table 3).

Table 3. Antibodies used in protein immunoblot analyses.

Primary antibodies		
Protein	**Catalog number**	**Producer**
GPx1	Ab22604	Abcam
mnSOD	Ab16956	Abcam
NFκB p65	Ab7970	Abcam
IκBα	Ab32518	Abcam
HSP70	ADI-SPA-810	Enzo Life Sciences
αB-crystallin	ADI-SPA-222	Enzo Life Sciences
Desmin	Ab6322	Abcam
Secondary antibodies		
Goat anti-mouse	31430	Thermo Scientific/Pierce biotechnology
Goat anti-rabbit	7074	Cell Signaling Technology

All antibodies were diluted in a 1% fat free skimmed milk and 0.05% TBS-t solution at room temperature. Membranes were washed in 0.05% TBS-t solution.

Bands were visualized using a 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).

ELISA

HSP27 was measured with an in house-made double antibody sandwich ELISA using a monoclonal capture antibody against HSP27 (25 ng/well; mouse-anti HSP27, Cat#ADI-SPA-800, Enzo Life Sciences) and a polyclonal detection antibody against HSP27 (rabbit-anti HSP27, Cat#ADI-SPA-803, Enzo Life Sciences). Horseradish peroxidase-conjugate as secondary antibody (Cat#RPN4301, Amersham Biosciences, GE Healthcare Life Sciences, Buckinhamshire, UK). The HSP27 assay was performed in high-binding polystyrene micro plates (Cat#3590, Costar Inc., Corning, NY, USA) using tetramethylbenzidine (TMB Solution, Cat#CL07, Calbiochem, EMD Biosciences) as substrate and 2 N sulfuric acid as stop solution. Recombinant HSP27 (Cat#SPP-715, Enzo Life Sciences) was used as standards (0.0975 – 25 ng ml-1). All samples were diluted 1:300 (cytosolic fraction) or 1:50 (cytoskeletal fraction) and analyzed in triplicates (CV <10%). The amount of HSP27 was determined using a filter photometer measuring optical density at 450 nm.

RT-qPCR

Total RNA was extracted from muscle biopsies using TRIzol reagent (Cat# 15596, Invitrogen, Life Technologies). Quantitative-PCR (q-PCR) analysis was performed in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For detailed information on the two techniques used, please refer to paper 2, 3 and 4.

In study 2, qPCR reactions was performed 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), and PerfeCTa SYBR Green FastMix (Cat#95072, Quanta Biosciences Inc., Gaithersburg, MD, USA). Target gene expression was calculated using GeNorm (Vandesompele *et al.*, 2002), making use of normalization factors determined from the two most stable reference genes, evaluated from data on the five frequently utilized reference genes β_2-microglobulin (β_2m), peptidylprolyl isomerase A (PPIA, cyclophilin A), β-actin (β-a), ubiquitin C (UBC), polymerase (RNA) II (DNA directed) polypeptide A (PolR2A), and ribosomal protein L32 (RPL32). Overall, PPIA and RPL32 was found to be most stable, with an M-value of 0.591, below the limit set by Vandesompele et al. (2002). The primers used for RT–qPCR analyses in paper 2 are listed in table 4.

Table 4. Human primer sequences used for RT-qPCR analyzes in study 2.

Gene name	Sense	Antisense
PPIA	GGTTTATGTGTCAGGGTGGTG	TCCCCATAGATGGACTTGC
β_2-m	GAGGCTATCCAGCGTACTCC	TCCATTCTCTGCTGGATGAC
RPL32	TTAAGCGTAACTGGCGGAAAC	GGCCCTTGAATCTTCTACGAAC
PolR2A	GGAGATCTTCACGGTGCTG	AGCCATCAAAGGAGATGACG
B-actin	ACCCCGTGCTGCTGAC	AACATGATCTGGGTCATCTTCTC
CRYAB	TCTCAGAGATGCGCCTGGAG	TCTGGGGAGAAGTGCTTCAC
HSPB1	TGGAGATCACCGGCAAGC	CGTGTATTTCCGCGTGAAGC
HSPB2	GCCGAGTACGAATTTGCCAA	TCAGGATCTCTTCTGGCAGGA
HSPB3	ATCGTGGACCTGAGGAAAACC	AAGTGGGATTTGCCTTCTCG
HSPD1	CGAGCCTTAATGCTTCAAGGTG	TCACTGTTCTTCCCTTTGGC
HSPA4	TACAGCTCTCCTCAGGATTTGC	CACTGGACACACTGAAAATGCC
HSP90AA1	CAAAGAAGGCCTGGAACTTCC	CGGTTTGACACAACCACCTTTTC
SOD1	TGAAGAGAGGCATGTTGGAGAC	AATAGACACATCGGCCACAC
SOD2	TTGGCCAAGGGAGATGTTACAG	TTAGGGCTGAGGTTTGTCCAG
GPx1	ATCAGGAGAACGCCAAGAAC	TTCACCTCGCACTTCTCGAAG

In paper 3 and 4, qPCR reactions were performed using Power SYBR Green RNA-to-Ct TM 1-step Kit (Cat# 4389986, Applied Biosystems) supplemented with forward and reverse primers. The Reverse Transcription step was performed by co-incubating the PCR primers with 6 ng of RNA and the MultiScribeTM Reverse Transcriptase (Cat#4311235, Invitrogen, Life Technologies) at 48 °C for 30 minutes in the presence of RNase Inhibitor. Thermocycling conditions were according to the recommendations of the manufacturer. Ct values for gene expression were calculated according to the comparative Ct method (Pfaffl, 2001). Relative quantification was performed by simultaneous quantification of GAPDH and 18S gene expression. The primers used for RT–qPCR analyses in paper 3 and 4 are listed in table 5.

Table 5. Human primer sequences used for RT-qPCR analyzes in study 3 and 4.

Gene name	Sense	Antisense
CRYAB	GTCAACCTGGATGTGAAGCA	TTTTCCATGCACCTCAATCA
HSPB1	GGACGAGCTGACGGTCAAG	AGCGTGTATTTCCGCGTGA
HSPA4	TTAAGTCCAAAATCCGTGCAT	CTGAAGCATTTGCACTCATCA
SOD2	CCCTGGAACCTCACATCAAC	GGTGACGTTCAGGTTGTTCA
GPx1	ACGATGTTGCCTGGAACTTT	TCGATGTCAATGGTCTGGAA

Histochemical staining

Histochemical analyses were done on eight-micrometre-thick cross sections cut in a microtome at 20°C (CM3050; Leica Biosystems GmbH, Wetzlar, Germany) and mounted on microscope slides (Superfrost Plus, Thermo Scientific, MA, USA).

The muscle sections were blocked for 30 min with 1% BSA (bovine serum albumin; Cat#A4503, Sigma Life Science, St Louis, MO, USA) and 0.05% PBS-t solution (Cat#524650, Calbiochem, EMD Biosciences) before incubated with primary antibodies listed in table 6.

Table 6. Antibodies used during immunohistochemical stainings

Primary antibodies		
Protein	**Catalog number**	**Producer**
HSP27	ADI-SPA-803	Enzo Life Sciences
HSP70	ADI-SPA-810	Enzo Life Sciences
aB-crystallin	ADI-SPA-222	Enzo Life Sciences
Desmin	Ab6322	Abcam
Myosin heavy chain 1	BA-D5	Gift from Prof. S. Schiaffino
Dystrophin	Ab15277	Abcam
Secondary antibodies		
Alexa fluor 594	A11005	Life Technologies
Alexa fluor 488	A11001	Life Technologies

Between stages, the sections were washed 3 x 5 min in 0.05% PBS-t solution. Muscle sections were then covered with a coverslip and glued with ProLong Gold Antifade Reagent with DAPI (Cat#P36935, Invitrogen Molecular Probes, Eugene, OR, USA) and left to dry overnight at room temperature. Muscle sections were visualized using a high-resolution camera (DP72, Olympus Corp., Tokyo, Japan) mounted on a microscope (BX61, Olympus Corp., Japan) with a fluorescence light source (X-Cite 120PCQ; EXFO Photonic Solutions Inc., Mississauga, ON, Canada).

Glycogen content in fibres was visualized by periodic acid-Schiff (PAS) staining (Cat#395-1, Cat#395-2, Cat#GHS-3; Sigma-Aldrich, Steinheim, Germany).

Staining intensities were analyzed using ImageJ with Fiji image processing package (http://fiji.sc/Fiji).

Muscle glutathione

Muscle GSH was analyzed by a commercial company (Vitas AS, Oslo, Norway) using a reagent kit originally intended for measurement of total homocysteine in plasma by HPLC (Cat#195-4075, Bio-Rad). The kit was modified and validated for quantification of total glutathione in tissues, with a detection limit (LOQ) of 0.04 mM. All samples were analyzed in triplicates (CV% < 2%).

Statistics

Study 1: A nonparametric Friedman test and Dunn's post hoc test were used to investigate changes over time for each leg. Differences between legs were analyzed using a nonparametric Wilcoxon matched-pairs signed-rank test. A Kruskal–Wallis test with Dunn's multiple comparisons test was used to investigate changes in fiber distributions for glycogen staining intensity. Correlations were tested using the Pearson product-moment correlation coefficient test. Outliers were identified using the Grubbs' test.

Study 2: A two-way ANOVA was used to evaluate the acute and long term effects of training (time) 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 response in untrained (first exercise bout) and trained (last training bout) skeletal muscles.

Study 3, 4: In both studies a two-way ANOVA was used to evaluate the effects of training (time) and supplementation, and a Holm-Sidak multiple comparisons test was applied for post hoc analyses.

For study 3 only: unpaired T-test was used to evaluate differences between groups in participant characteristics and variables for exercise intensity in the acute experiment. Correlations were tested using the Pearson product-moment correlation coefficient test. Effect sizes for mRNA data was calculated and reported using Cohen's *d*. An effect size of 0.2 was considered small, 0.5 medium, 0.8 large and 1.3 very large. The level of significance was set to $P < 0.05$ for all studies. All results are presented as mean±standard deviation. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses.

Results and discussion

Heat shock proteins accumulate in cytoskeletal structures (study 1, 2 and 3)

Prior to this study, heat shock proteins (HSP) have been shown to increase in cytoskeletal structures after high-force eccentric exercise, and in animal models by exposure to ischemic conditions (Armstrong et al. 1999; Golenhofen et al. 2004; Koh and Escobedo, 2004; Koh, 2002; Paulsen et al. 2009; Yoshida et al. 1999). In the first hour after low load blood flow restricted resistance exercise (BFRE), HSP27 and αB-cystallin decreased in the cytosolic cell compartment, concomitantly with an increase in cytoskeletal structures (figure 3). αB-crystallin seems to translocate to cytoskeletal structures from membrane and cytosolic compartments, as the αB-cystallin levels decreased in both these cell compartments (figure 3).

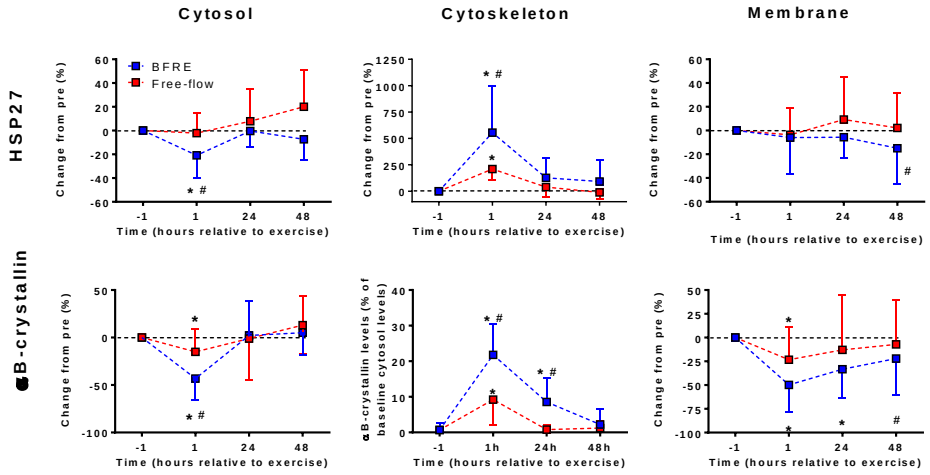

Figure 3. HSP27 and αB-crystallin levels in the cytosolic, cytoskeletal and membrane cell fractions. HSP27 and αB-crystallin are expressed as changes relative to pre-exercise levels except for αB-crystallin cytoskeletal levels which are expressed as relative levels compared to baseline cytosolic levels. Blue illustrates HSP levels in the blood flow restricted leg (BFRE-leg) and red for the free-flow leg. Values are presented as mean and standard deviations. * Different compared to pre-exercise (-1 hour) levels. # Differences between legs.

HSP70 on the other hand, increased in cytoskeletal structures 24 hours after exercise, and remained elevated at 48 hours after the BFRE bout (figure 4). Since BFRE is known to induce both high metabolic and ischemic stress (Suga *et al.*, 2009, 2012; Kacin & Strazar, 2011; Karabulut *et al.*, 2014), it is likely that this occurs primarily as a result of these stresses – combined or separately. Increases in cytoskeletal HSP were mainly seen in the BFRE leg, with no or little increases in the free-flow leg; which conducted the same training volume (load and repetitions), but with normal (intact) blood flow to the exercised limb. This response was observed in muscles unaccustomed to BFRE, but not in muscles accustomed to this type of exercise (after 12 weeks of training) (figure 5).

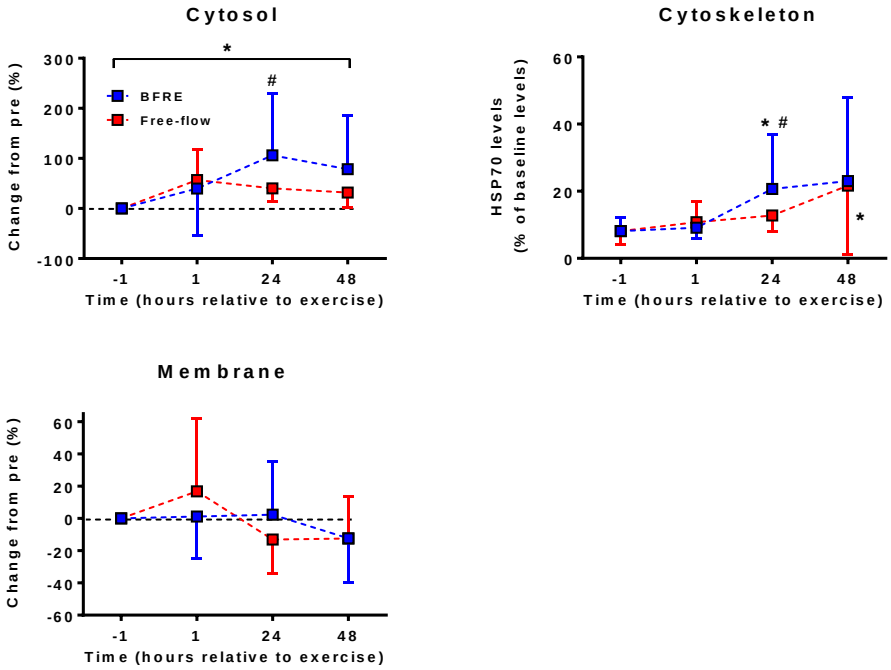

Figure 4. HSP70 levels in the cytosolic, cytoskeletal and membrane cell fractions. Cytosolic and membrane HSP70 is expressed as changes relative to pre-exercise levels. Cytoskeletal HSP70 levels are expressed as relative levels compared to baseline cytosolic levels. Blue illustrates HSP levels in the blood flow restricted leg (BFRE-leg) and red for the free-flow leg. Values are presented as mean and standard deviations. * Different compared to pre-exercise (-1 hour) levels. # Differences between legs.

In study 2, the HSP responses after BFRE were compared to the response after a traditional, heavy load strength training sessions (HLS). Before the training period, BFRE and HLS induced similar HSP responses; both exercises increased αB-crystallin and HSP27 content in cytoskeletal structures (figure 5). However, if the muscles are accustomed to BFRE and HLS, the trained muscles did not show the same translocation to cytoskeletal structures as in the untrained muscles. This can be the results of training adaptations where weak structures in the cytoskeleton are strengthened. But most importantly, higher resting levels of HSPs (HSP27) in the cytoskeleton might also have contributed to the improved exercise tolerance as higher content of HSP in these structures will increase the protection against future damage.

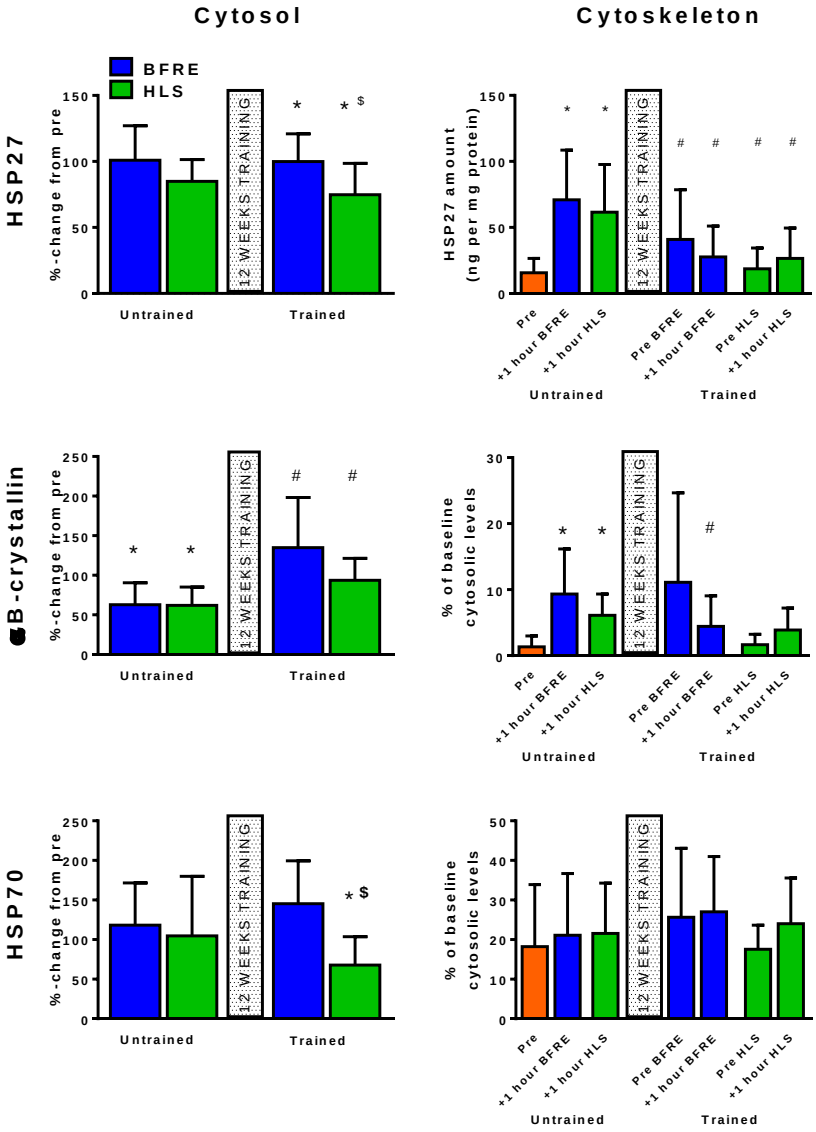

Figure 5. HSP27, αB-crystallin and HSP70 levels in the cytosolic and cytoskeletal cell fractions in untrained and trained state (after 12 weeks of BFRE or HLS training) one hour after an exercise session. Cytosolic HSP levels are expressed as changes relative to pre-exercise levels. Cytoskeletal HSP27 levels are expressed as ng HSP27 per mg protein. Cytoskeletal αB-crystallin and HSP70 levels are expressed as relative levels compared to baseline cytosolic levels. Blue illustrates HSP levels in the blood flow restricted leg (BFRE-leg), green for the heavy load strength training leg (HLS-leg) and orange for pre training (baseline) values. Values are presented mean and standard deviations. * Different compared to pre-exercise levels. # Differences between legs. $ Different compared to the first training session (before the training intervention).

BFRE and HLS induce different stresses to the muscle during exercise, and it is therefore plausible that the mechanism behind the translocation to cytoskeletal structures differs between the two exercises. Indeed, HSPs translocate and accumulates in cytoskeletal structures after high load eccentric exercise (Paulsen *et al.*, 2007), which induce high mechanical forces and relative low metabolic stress to the muscle. On the other hand, it is demonstrated in animal models that HSP translocate to cytoskeletal structures after ischemic conditions (Yoshida *et al.*, 1999; Armstrong *et al.*, 1999; Golenhofen *et al.*, 2004). Since BFRE typically involves considerable ischemia, and low mechanical forces, it is plausible that translocation to cytoskeletal structures is related to another type of stress than what's occurring during HLS. And due to the recruiting pattern of BFRE, it specifically stress the type 1 fibers (Wernbom *et al.*, 2009), in contrast to HLS, which mainly stress the type 2 fibers (Folkesson *et al.*, 2008).

Overall, due to the biological functions of the HSPs, the translocation of HSPs to cytoskeletal structures indicates partly or completely denatured proteins located the cytoskeleton. These results show that BFRE and HLS are capable to induce cell stress to cytoskeletal structures. This is in contrast to the belief that BFRE does not induce pronounced cellular stress and consequently not induce muscle damage (Loenneke *et al.*, 2014). The functional importance of this translocation seem apparent since the increases of αB-crystallin and HSP27 in the cytoskeletal structures correlated with reductions in muscle force after BFRE 24 hours after exercise, something which is also observed after high intensity eccentric exercise (Paulsen *et al.*, 2007, 2009).

Long lasting reductions in muscle force after exercise is considered an indirect, but functional sign of muscle damage since it correlates with the number of damaged muscle fibers after exercise (Koh & Brooks, 2001; Raastad *et al.*, 2010; Paulsen *et al.*, 2012*b*). However, it has also been suggested that reduction in muscle force after BFRE is only related to fatigue in the muscle, as many studies only observe short term (<24 h) reductions in force (Loenneke *et al.*, 2014). As both might be true for BFRE, it is important to point out that there are large differences whether the exercise protocol is conducted to failure or a pre-set number of repetitions (not to failure). Furthermore, is seems to be of great importance whether the muscle is accustomed to this kind of exercise, as evident from the blunted HSP response observed in the training study.

As presented earlier, the HSP response, with the characteristic translocation of HSPs to damaged or stressed areas, is widely studied after resistance type of exercises, and not representable for all types of exercises. Indeed, the degree of HSP translocation/responses is usually dependent on type and intensity of the exercise. After a single bout of high intensity interval/endurance exercise we found decreased cytosolic levels of αB-crystallin and HSP27, with a slight tendency to increased HSP27 amounts in cytoskeletal structures (no detectable levels for αB-crystallin). High intensity interval training has previously been shown to increase αB-crystallin and HSP60 levels in males after 6 weeks of training (Morton *et al.*, 2006), so it is likely that this type of exercise will induce translocation to damaged areas within the muscle cell. Interestingly, the participants in our study were already trained. But most importantly the decrease in cytosolic αB-crystallin and HSP27 only occurred in muscles of individuals who had been supplemented with high doses of vitamin C and E in the weeks (and immediately) prior to the standardized exercise session. This indicates that there might have been some effect of the supplements on the training adaptation during the first weeks of exercise, so the stress response to exercise was not as well controlled as in the placebo group. A speculation is

that the placebo group tolerated stress from the intervals better than the vitamin C and E group because of more adequate training adaptations in this period. More precisely, mitochondrial adaptations to the endurance training was hampered in the vitamin C and E group, as previously reported (Paulsen *et al.*, 2014). Lack of mitochondrial adaptation might have kept the exercise-induced stress stable even though the exogenous antioxidants were increased by supplementation.

To summarize, HSPs translocate acutely to damaged or highly stressed areas within the cell after exercise, mostly to cytoskeletal structures. It is common beliefs that exercise intensity, and exercises that induce disruptions in the myofibrils, induce large translocations. Here we show that translocation also occurs after low-load exercises like BFRE, which mainly induce metabolic- and ischemic stress. This translocation seems to mainly occur in the type 1 fibers, as presented in the next section. When the muscles get accustomed to BFRE, the HSP stress response is absent. This is can be explained by strengthened structures and higher levels of HSPs (HSP27) in the cytoskeletal structures. Further, high doses of antioxidants (vitamin C and E), might affect training adaptations that could affect stress levels in the exercised muscle, and result in larger HSP responses.

Fiber specific responses after low load BFRE (study 1)

Based on electromyography (EMG) data, Wernbom et al. (2009) speculated that due to the low loads, type 1 fibers do most of the job in the first repetitions, but because of rapid development of fatigue from BFRE, type 2 fibers are gradually recruited during the first set. Because of the lack of recovery between sets with BFRE, type 2 fibers are probably recruited earlier in the following sets (Moritani *et al.*, 1992; Wernbom *et al.*, 2009). Extensive recruitment of type 2 fibers during BFRE was confirmed by Krustrup et al. (2009), but because of the earlier recruitment, it is reasonable to assume that more stress is put on type 1 fibers during BFRE because they are exposed to considerably longer durations of exercise stress.

By the use of immunohistochemical methods, it was shown in study 1 that the HSP70 response was more pronounced in type 1 fibers after BFRE than in the work matched control (figure 6). For αB-crystallin, increased staining in type 1 fibers was seen after both BFRE and in the free-flow leg (figure 6). Increased staining intensity indicates increased HSP binding, mainly in cytoskeletal structures. The increased HSP70 staining in type 1 fibers is in agreement with observations after submaximal isometric contractions, as reported by Tupling et al. (2007). The increased HSP70 staining in type 1 fibers indicates that these fibers are highly affected by BFRE, which is in contrast to findings after eccentric exercise where increased HSP staining were predominantly found in type 2 fibers (Paulsen et al. 2009). However, as type 2 fibers seem to be recruited later than type 1 fibers with BFRE (as discussed earlier), the longer durations of exercise stress probably contributed to this distinct fiber specific pattern.

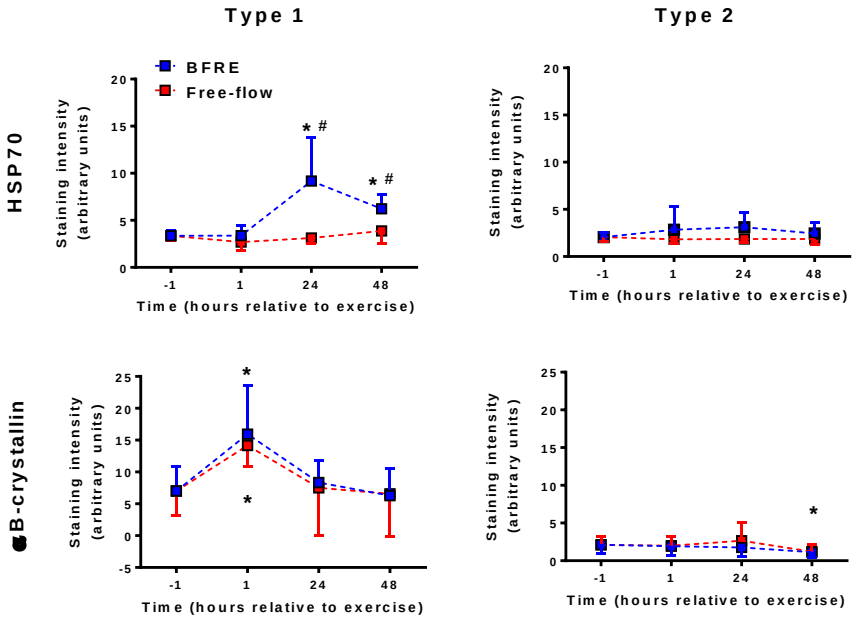

Figure 6. HSP70 and αB-crystallin staining intensity in type 1 and type 2 muscle fibers. Blue illustrates HSP staining intensity in the blood flow restricted leg (BFRE-leg) and red for the free-flow leg. Values are presented as means and standard deviations. * Different compared to pre-exercise intensities. # Differences between legs.

In support of the hypothesis that type 1 fibers were more stressed than type 2 fibers, we also observed reduced glycogen content in type 1 fibers. The reduction in glycogen was additionally more prominent in the BFRE leg than in the free-flow leg. Previous studies have shown reduced glycogen content and resynthesis in the days after eccentric exercise (O'Reilly *et al.*, 1987; Costill *et al.*, 1990; Doyle *et al.*, 1993). Although we did not observe similar signs of muscle damage after BFRE as found after eccentric contractions, depleted glycogen in muscle fibers the first days after exercise is an indirect sign of impaired glycogen resynthesis. It has been suggested that this is caused by decreased GLUT4[16] in the cell membrane, and impaired insulin action (Asp *et al.*, 1995, 1996). In fact, Wernbom et al. (2012) showed increased sarcolemmal permeability, which might be a sign of damage to the cell membrane. This would inhibit effective GLUT4 translocation to the cell membrane, but also impair insulin signaling.

Interestingly, fibers that were glycogen depleted (or had low glycogen levels) also displayed a strong staining intensity against HSP70 48 h after exercise (figure 7). This may indicate that the increased HSP70 levels were linked to reduced energy availability in the fibers. Actually, HSPs have been shown to increase when glucose availability is low (Febbraio & Koukoulas, 2000*b*). In contrast to our findings, Tupling et al. (2007) reported preferentially glycogen depletion in type 2 fibers concomitantly with increased HSP70 content in type 1 fibers after isometric contractions.

[16] Glucose transporter type 4: responsible for insulin and exercise mediated glucose uptake in skeletal muscles.

Intriguingly, in study 1, the glycogen content in type 1 fibers appeared to stay low for at least 48 h in the BFRE leg. Altogether, the fiber specific HSP responses (αB-crystallin and HSP70) and the acute and prolonged glycogen depletion strongly indicate that type 1 fibers were more stressed than type 2 fibers during low-load BFRE.

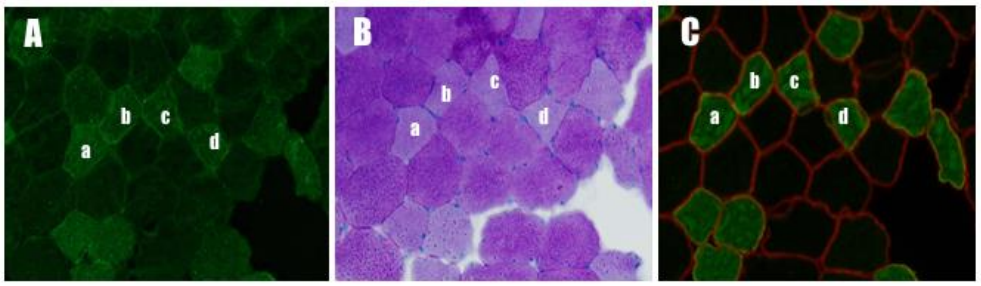

Figure 7. Neighboring muscle cross sections stained with antibodies against HSP70 (A), glycogen content stained with PAS staining (B), and antibodies against myosin heavy chain 1 (type 1 muscle fibers; C). Muscle fibers displaying HSP70 staining co-localizes with fibers displaying low glycogen content and type 1 muscle fibers.

Heat shock proteins and training adaptations (study 2, 3 and 4)

As presented in table 1, there is well documented that HSPs increase with systematic and regular strength and endurance training. BFRE is usually conducted with low intensity (low loads), which according to previous studies is an important factor for the increase observed in HSP levels after strength training (table 1). However, due to the acute HSP response seen after BFRE (described above), low load BFRE should also have a great potential to increase resting levels of muscle HSPs. However, after 12 weeks of BFRE no changes in αB-crystallin, HSP27 or HSP70 were observed (figure 8), despite an increase in muscle strength of ~12% (and size; unpublished). Further, the lack of increases was not only observed after BFRE, but also after HLS. The absence of changes may be a result of the training frequency conducted in the study. Two sessions per week might not be sufficient to increase HSP content, and more frequent exercise bouts might be needed. As listed in table 1, most (human) studies investigating changes in HSP content after a period of training, is conducted at least three times per week, with the exception of Gjøvaag et al. (2006), which conducted 2-3 (2.5) exercise sessions per week with exercises that only consisted of either concentric or eccentric contractions. As mentioned earlier, there might also be some sex-related issues related to the lack of changes in HSP levels, as females show lower HSP levels and reduced response to training (Morton *et al.*, 2009*b*).

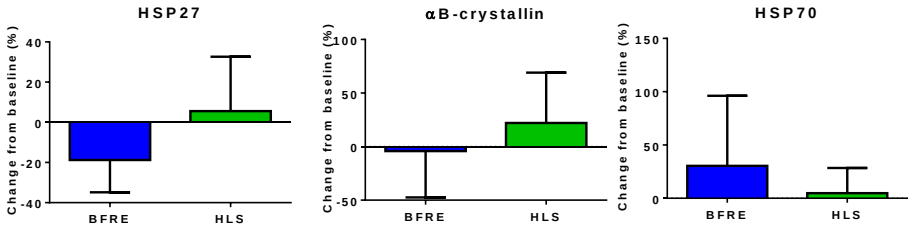

Figure 8. Changes in resting levels of HSP27, αB-crystallin and HSP70 levels after 12 weeks of BFRE or HLS training. HSP levels are expressed as changes from pre-training levels. HSP27 levels are a sum of HSP27 protein content in the cytosolic and cytoskeletal cell compartment combined. Blue illustrates HSP levels in the blood flow restricted leg (BFRE-leg) and green for the heavy load strength training leg (HLS-leg). Values are presented mean and standard deviations.

Unchanged HSP levels is in contrast to the fact that gene expression of *CRYAB* and *HSPB1* (which codes for αB-crystallin and HSP27, respectively), increased acutely after both the first and last exercise bout (figure 9), whereas *HSPA4* (codes for HSP70) was only higher after the last exercise session (after 12 weeks of training; figure 9). It cannot be ruled out that an increase in HSP levels was present during the first weeks of training, as these exercises were still new for the muscle cells. If this was the case, a downregulation must have occurred when the muscle got better adapted to the exercises, and the HSP levels stabilized to pre-exercise levels. However, increased HSP27 content in the cytoskeletal structures indicate increased protection of these structures, and probably contributed to increased exercise tolerance.

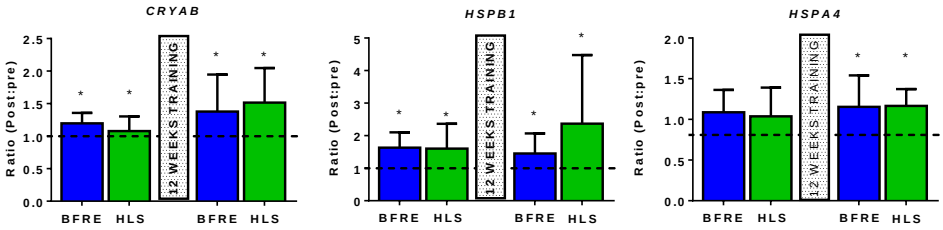

Figure 9. Changes in *CRYAB*, *HSPB1* and *HSPA4* mRNA levels/expression after the first (left set of graphs) and last exercise bout (right set of graphs) of BFRE and HLS. mRNA levels is expressed as ratio (post:pre). Blue illustrates HSP levels in the blood flow restricted leg (BFRE-leg) and green for the heavy load strength training leg (HLS-leg). Values are presented mean and standard deviations. *: different from pre exercise expression (P<0.05).

The effects of vitamin C and E supplementation on HSPs have been studied in a few previous studies (see introduction for details). In trained males and females antioxidant supplementation did not affect resting levels of the HSPs, after neither endurance nor HLS training (study 3 and 4; figure 10). Interestingly, muscle αB-crystallin and HSP27 content decreased in both the vitamin C and E and placebo supplemented group after 11 weeks of endurance training. For HSP70 the same decrease was not observed. However, two participants (one in each group) displayed a large increase in HSP70 content after the training intervention. By removing these data points from the statistical analyses, a decrease was observed in both groups. A decrease in αB-crystallin, HSP27 and HSP70 content could indicate less stimulatory stress on the muscle to maintain the HSP levels, as the demand for HSPs is reduced, and the muscles get accustomed to the endurance training in the study. It is also plausible that reductions in HSP levels were a result of more regular and systematic training sessions. While more sporadic training, with longer periods without training or periods of very frequent training (followed by periods of fewer training sessions) would be more stressful for the muscles, thus, lead to higher levels of HSPs.

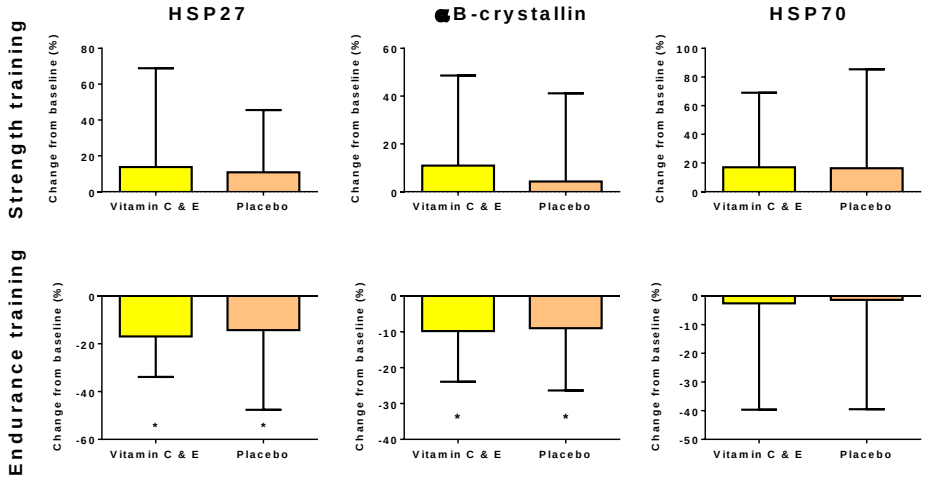

Figure 10. Changes in resting levels of HSP27, αB-crystallin and HSP70 levels after 10 weeks of HLS (upper panel of graphs) and 11 weeks of endurance training (lower panel of graphs) in response to vitamin C and E supplementation. Yellow illustrates HSP levels in the vitamin C and E group and pink for the placebo group. Values are presented mean and standard deviations. *: different from pre exercise levels (P<0.05).

Interestingly, when including a time point midway into the training period (pre-sample from the acute exercise study conducted 5-6 weeks into the training period) the vitamin C and E group had unchanged HSP27 levels, whereas the placebo group had reduced HSP27 levels already at this time point. This finding might indicate that more exercise stress was generated in the vitamin C and E group than in the control group during the initial 5-6 weeks of training. Nevertheless, because the HSP27 levels were decreased similarly in both groups after 11 weeks of endurance training, it seems like this effect was temporary and exclusively related to the supplementation in the initial weeks of training. The hypothesis that more stress was generated in the vitamin C and E group was supported by the gene expression observed after the acute midway exercise bout. Ninety minutes after the exercise bout, larger expression of *CRYAB* and *HSPA4* was observed in the group that had been supplemented with vitamin C and E (analyzed using effect size calculations). Higher gene expression, could, together with the difference observed in the acute αB-crystallin and HSP27 translocation (described earlier), suggest that more stress was generated during the acute interval session in the vitamin C and E group, compared to the placebo group. In support of this, results from a recent study showed that animals supplemented with high doses of vitamin E had less tolerance to oxidative stress than animals fed with a placebo (Venditti *et al.*, 2014). Here, the researchers found that antioxidant supplementation hampered mitochondrial biogenesis, which also affected H_2O_2-release from the mitochondria.

On the other hand, in study 4 we observed no changes in neither αB-crystallin, HSP27 nor HSP70 levels after 10 weeks of HLS training. Hence, there were no effects of vitamin C and E supplementation on the HSP levels. An explanation to the lack of increases could be that our participants were trained before entering the study and already had high levels of HSPs, thus less

potential to increase HSP content in trained muscles (Smolka *et al.*, 2000; Gjøvaag & Dahl, 2006). However, HSP levels can increase in highly trained athletes, but this can only be achieved with very intensive exercise (Liu *et al.*, 2000, 2004). Thus, further increases in HSP can only be achieved if the training were conducted at even higher intensity. Taken together, the results presented here is an indication that changes in HSP levels is not as straight forward as first assumed. Since muscle cells already have high content of HSPs (Larkins *et al.*, 2012*a*), increases in HSP content can reflect a state where the cells need to overreach the content of HSPs after exercise, as a precautionary-mechanism. Meanwhile, when the exercise is repeated the HSP content stabilizes to the already high baseline levels. Thus, large increases in HSPs can display too intense training/exercise where the muscle cells are fighting to maintain its intracellular homeostasis – something which won't be optimal in the long term.

To summarize, previous studies have reported that HSP content increases in skeletal muscles as an adaptation to training. Here, no increases were observed after low load BFRE or HLS training, in both untrained and trained volunteers (study 2 and 4). Surprisingly, decreased HSP levels were observed after endurance training in trained individuals (study 3). As previously reported, antioxidant supplementation might affect HSP levels. However, no effects of vitamin C and E supplementation were observed on resting HSP levels after HLS or endurance training (study 3 and 4). On the other hand, Vitamin C and E supplementation affected HSP gene expression during the initial weeks of training, probably by inhibiting adaptations to endurance training, and thus hamper the training induced improvements in exercise tolerance.

Antioxidant systems and training adaptations (study 2, 3 and 4)

Exercises that relies heavily on anaerobic energy supply, such as strength and sprint exercises, has a great potential to produce RONS (Liu *et al.*, 2005; Morales-Alamo & Calbet, 2014) and thus also potentially induce oxidative damage. In previously untrained females (study 2), GPx1, *SOD1 and SOD2* gene expression did not increase acutely after the first or last exercise bout of neither BFRE nor HLS. However, resting *GPx1* and *SOD2* gene expression were lower in trained state than untrained state. This lack of acute increases in antioxidant gene expression was reflected in no changes in GPx1 and mnSOD content after the training intervention (figure 11). The most likely explanation for these results is that the exercise *per se* did not result in considerable and long-term alterations in redox homeostasis, and consequently there was no increased demand for GPx1 and mnSOD. Since the training was conducted twice per week, the repeated accumulation of stress from training did probably not exceed the levels that would induce changes in the enzyme content. Potentially more frequent exercises per week could accumulate more stressful insults to induce changes in these antioxidant enzymes. In addition to the training, there is a possibility that the baseline endogenous antioxidant content was at sufficient levels to cope with the exercise induced RONS production during the training intervention. Furthermore, although BFRE has the potential to induce oxidative damage through the reduced blood flow and/or the reperfusion[17] of blood (Adachi *et al.*, 2006; Westman *et al.*, 2007), it is still unknown whether BFRE leads to considerable oxidative damage as previous studies did not find changes in markers for oxidative damage (Takarada *et al.*, 2000; Goldfarb *et al.*, 2008). However, these indirect markers of oxidative damage/stress were measured in

[17] Reperfusion is when the blood flow returns to the affected tissue after being restricted/reduced

blood samples, and after exercises with relative small volume of muscle mass analysis in blood do probably not represent what's happening in the exercising muscles.

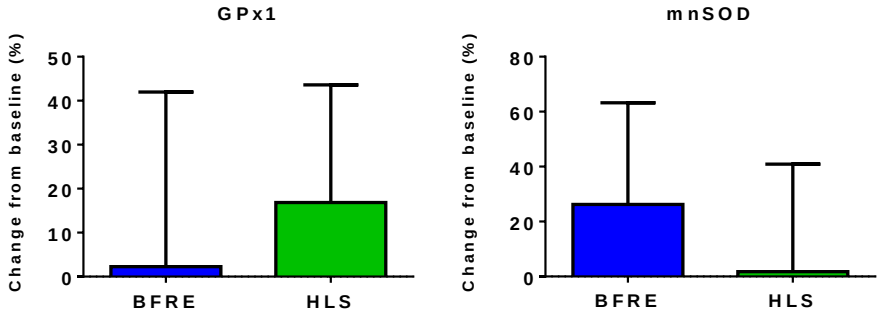

Figure 11. Changes in resting levels of GPx1 and mnSOD levels after 12 weeks of BFRE or HLS training. Blue illustrates HSP levels in the blood flow restricted leg (BFRE-leg) and green for the heavy load strength training leg (HLS-leg). Values are presented mean and standard deviations.

Also in trained males and females (study 4), no changes in *SOD2* or *GPx1* gene expression were observed after HLS exercise. After 11 weeks of endurance training, previously trained individuals had unchanged muscle content of mnSOD and GSH compared to pre training content (figure 12), this was also reflected in unchanged *SOD2* gene expression after the acute exercise bout. Unchanged levels happened although the exercise consisted mostly of high intensity interval training, which is previously shown to increase these antioxidants in skeletal muscle (table 2 and 3). Surprisingly, GPx1 content decreased over the training period (figure 11), in addition to unchanged acute *GPx1* expression (slight tendency to increased expression). Decrease in GPx1 is in agreement with Gliemann et al. (2013), who reported that eight weeks of high-intensity endurance training reduced GPx1 in skeletal muscles of initially inactive elderly men. These results are in contrast to most human studies, which report no change in muscle GPx1 levels after endurance training (table 2). The exact cause for the decrease is hard to explain, but it might be linked to improvements in mitochondrial function, as mitochondria from trained animals have decreased H_2O_2 production (Venditti *et al.*, 2014). In trained individuals, it is plausible that further increases in both mnSOD and GPx1 could alter intracellular RONS levels to a degree that could hamper RONS signaling and, thus, prevent normal cellular functions and further adaptations to training.

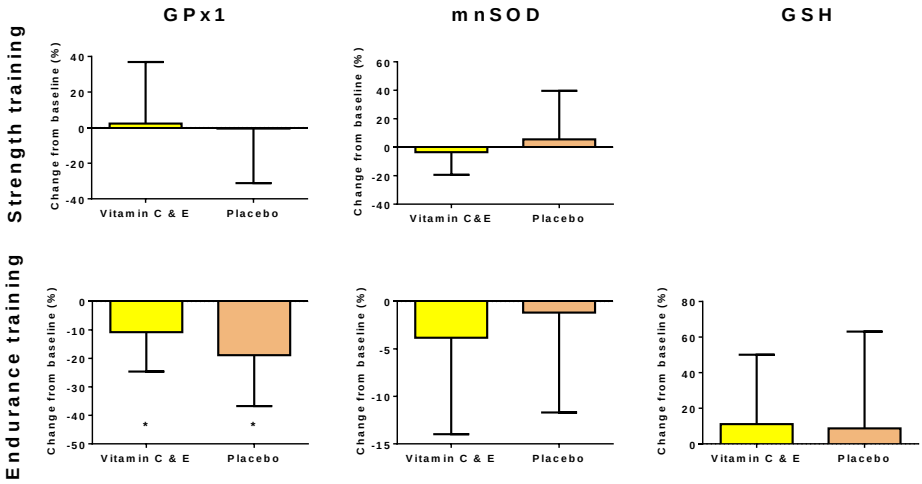

Figure 12. Changes in resting levels of GPx1, mnSOD and GSH levels after 10 weeks of HLS (upper panel of graphs) and 11 weeks of endurance training (lower panel of graphs) in response to vitamin C and E supplementation. Yellow illustrates HSP levels in the vitamin C and E group and pink for the placebo group. Values are presented mean and standard deviations. *: different from pre exercise levels (P<0.05).

As mentioned earlier, antioxidant supplementation might affect adaptions in the antioxidant system, as previously reported both in blood and in skeletal muscles. However, in studies 3 and 4, vitamin C and E supplementation did not have any effects on adaptations in the antioxidant systems; neither negative nor positive (figure 11).

To summarize, several studies have previously reported that the endogenous antioxidants increase with training, especially the SODs. Here, no increases were observed after BFRE, HLS or endurance training. On the contrary; in pre-trained males and females muscle GPx1 decreased in response to endurance training. Decreased GPx1 levels might indicate improvements in mitochondrial function. Further, vitamin C and E supplementation did not affect these variables in the trained muscle.

Summary

The purpose of this doctoral thesis was to investigate the acute responses to exercise and adaptations to training in heat shock proteins and endogenous antioxidants.

The main findings were:

1. BFRE can induce a pronounced acute HSP response, especially in type 1 fibers. This response was seen as a rapid translocation of the small HSPs (αB-crystallin and HSP27) to cytoskeletal structures, and a slower accumulation of HSP70 to the same structures. This occurred together with glycogen depletion (in type 1 fibers). Additionally, this response was similar to what was observed after heavy load strength training. The HSP-response was only observed when the participants were unaccustomed to the exercise. The blunted HSP translocation to cytoskeletal structures in the trained muscle is possibly related to increased HSP27 content in these structures after 12 weeks of training together with a general strengthening of weak structures. This shows that the HSP are highly capable of responding to exercises performed with low loads which are considered less damaging than exercises with high loads.
2. HSP (HSP27, αB-crystallin and HSP70) content did not change after 10-12 weeks of systematic BFRE or heavy load strength training neither in muscles of previously trained or untrained volunteers. This can be related to the initial training status of the participants, sex-related differences in training adaptation and training volume. 11 weeks of endurance training decreased HSP27 and αB-crystallin content of previously trained male and females.
3. Vitamin C and E supplementation did not affect adaptations in HSP content after heavy load strength training or endurance training. However, increased exercise stress from a high intensity session of endurance exercise, shown by higher HSP gene expression and translocation of small HSPs to cytoskeletal structures, was observed in the vitamin C and E supplemented group after the initial weeks of training. Consequently, less tolerability towards exercise-induced stress in the vitamin C and E group could in the long-term, or during periods of very intense training, delay recovery and cause suboptimal gains in physical performance.
4. The endogenous antioxidants GPx1, mnSOD and GSH (not measured after BFRE-training) did not change after 10-12 weeks of systematic BFRE or heavy load strength training neither in muscles of previously trained or untrained volunteers. However, it is plausible that the lack of alterations in these antioxidants is related to an initial well-developed and efficient endogenous antioxidant system or the training frequency conducted. 11 weeks of endurance training decreased GPx1 levels in previously trained males and females. This can be related to increased physical fitness in the participants, with better mitochondrial function.
5. Vitamin C and E supplementation had no influence on GPx1, mnSOD and GSH content after heavy load strength training or endurance training.

Conclusion

The goal of the present doctoral thesis was to investigate the acute stress responses and training adaptations in heat shock proteins and antioxidant systems in human skeletal muscles (*m. vastus lateralis*) after exposure to different exercise stresses. In relation to our initial hypothesis the following was confirmed/not confirmed:

1. Low load blood flow restricted exercise (BFRE) will cause a larger acute HSP (HSP27, αB-crystallin and HSP70) translocation to cytoskeletal structures than work-matched free flow exercise.
   Confirmed
2. Low load BFRE will cause the same HSP (HSP27, αB-crystallin and HSP70) translocation to cytoskeletal structures as heavy load strength training.
   Confirmed
3. The acute HSP response with low load BFRE will be larger in type 1 than in type 2 fibers.
   Confirmed
4. Low load BFRE and heavy load strength training will induce an acute upregulation in HSP and antioxidant gene expression after exercise.
   Confirmed: *CRYAB*, *HSPB1*, *HSPA4*, *HSP90AA1*
   Not confirmed: *HSPB2*, *HSPB3*, *SOD1*, *SOD2*, *GPx1*
5. The acute HSP response after exercise will be reduced after 12 weeks of BFRE and heavy load strength training.
   Confirmed
6. 12 weeks of BFRE and heavy load strength training will increase HSPs (HSP27, αB-crystallin and HSP70) content in skeletal muscle.
   Not confirmed
7. 12 weeks BFRE and heavy load strength training will increase endogenous antioxidant (mnSOD and GPx1) content in skeletal muscle.
   Not confirmed
8. Vitamin C and E supplementation will blunt increases in HSP and antioxidant gene expression by blunting NFκB activation after a single session of high intensity interval running exercise.
   Not confirmed
9. Vitamin C and E supplementation will reduce the training induced increases in muscle HSP content (HSP27, αB-crystallin and HSP70) after 11 weeks of high intensity endurance training.
   Not confirmed
10. Vitamin C and E supplementation will reduce the training induced increases in endogenous antioxidant content (GSH, mnSOD and GPx1) in skeletal muscle after 11 weeks of heavy load strength training.
    Not confirmed
11. Vitamin C and E supplementation will blunt acute increases in muscle HSP (*HSPB1, CRYAB* and *HSPA4*) and antioxidant (*SOD2* and *GPx1*) mRNA expression by reduced NFκB activation after a single session of heavy load strength training.
    Not confirmed

12. Vitamin C and E supplementation will reduce the training induced increases in muscle HSP content (HSP27, αB-crystallin and HSP70) after 10 weeks of heavy load strength training. **Not confirmed**
13. Vitamin C and E supplementation will reduce the training induced increases in muscle antioxidant content (GSH, mnSOD and GPx1) after 10 weeks of heavy load strength training. **Not confirmed**

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Paper 1

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Acute response and subcellular movement of HSP27, αB-crystallin and HSP70 in human skeletal muscle after blood-flow-restricted low-load resistance exercise

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Abstract

Aim: Heat-shock proteins (HSP) are important chaperones for stressed and damaged proteins. Low-load blood-flow-restricted resistance exercise (BFRE) is generally believed not to induce significant muscle damage, but is hitherto unverified with intracellular markers. Consequently, the aim of this study was to investigate the HSP response after BFRE in human skeletal muscle.

Methods: Nine healthy volunteers performed five sets to failure of unilateral knee extension at 30% of 1RM with partial blood-flow restriction. The contralateral leg performed the same work with free blood flow. Muscle biopsies were collected before exercise, 1, 24 and 48 h after exercise and analysed for HSP27, αB-crystallin, HSP70, desmin, glycogen content and myosin heavy chain by immunohistochemistry, ELISA and western blotting.

Results: One hour after exercise, HSP27 and αB-crystallin levels were reduced in the cytosolic and increased in the cytoskeletal fraction in the BFRE leg. HSP70 showed a delayed response and was increased over 48 h in the BFRE leg. Immunohistochemical analyses showed higher staining intensity of HSP70 in type 1 fibres in the BFRE leg at 24 and 48 h post-exercise. PAS staining showed decreased glycogen levels after BFRE, and interestingly, glycogen was still depleted 48 h after exercise in the same fibres displaying high HSP70 staining (type 1 fibres).

Conclusion: Translocation of HSP27 and αB-crystallin from cytosol to cytoskeletal structures indicates that cytoskeletal proteins are stressed during BFRE. However, overt signs of myofibrillar disruptions were not observed. Interestingly, the stress response was more pronounced in type 1 than in type 2 fibres and coincided with low glycogen levels.

Keywords glycogen staining, muscle damage, occlusion training, stress proteins.

Low-load resistance training with blood-flow restriction has gained interest in the recent years because it has been shown to increase strength and induce muscle hypertrophy to a similar extent as conventional heavy load strength training (Wernbom *et al.* 2008). Because blood-flow-restricted resistance exercise (BFRE) is performed with low loads, usually <50% of 1 repetition maximum (1RM), it is suggested not to

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give any considerable degree of muscle damage, as indicated by unaltered plasma levels of creatine kinase in one study (Takarada *et al.* 2000). On the other hand, two studies have demonstrated reduced maximum force-generating capacity and prolonged recovery after BFRE (Umbel *et al.* 2009, Wernbom *et al.* 2012), which both indicate some degree of muscle damage (Paulsen *et al.* 2012).

A characteristic response to muscle-damaging eccentric exercise is myofibrillar disruptions (Paulsen *et al.* 2012) and intracellular accumulation of heat-shock proteins (HSPs) in the damaged areas (Koh & Escobedo 2004, Paulsen *et al.* 2009, Jørgensen *et al.* 2013). Heat-shock proteins 27 and 70 (HSP27 and HSP70) and αB-crystallin have been identified as important HSPs for repair and stabilization of stressed and damaged proteins (Noble *et al.* 2008). In the unstressed state, HSPs are primarily found free and unbound in the cytosolic cell compartment. However, upon stress such as muscle damage, they translocate, bind to and accumulate in stressed and damaged structures, for example the Z-discs (Koh 2002, Paulsen *et al.* 2007). Thus, histological detection of the small HSPs appears to be an effective way to locate damaged or highly stressed structures.

Interestingly, translocation and accumulation of HSPs in cytoskeletal structures have also been demonstrated in cardiac and skeletal fibres after ischaemic conditions (Armstrong *et al.* 1999, Yoshida *et al.* 1999, Golenhofen *et al.* 2004). This indicates that these cellular structures can be highly stressed or damaged even without the mechanical stress from high-force muscle contractions. BFRE typically involves brief ischaemia (Kacin & Strazar 2011), and the metabolic stress may be pronounced (Suga *et al.* 2009). Based on electromyography data, Wernbom *et al.* (2009) speculated that due to low loads, type 1 fibres do most of the job in the first repetitions, but due to the rapid development of fatigue with BFRE, type 2 fibres are gradually recruited during the first set. Furthermore, because of lack of recovery between sets with BFRE, type 2 fibres are probably recruited earlier in the subsequent sets (Moritani *et al.* 1992, Wernbom *et al.* 2009). Extensive recruitment of type 2 fibres during BFRE was confirmed by Krustrup *et al.* (2009), but because of the earlier recruitment, it is reasonable to assume that more stress is put on type 1 fibres during BFRE. Consequently, it is intriguing to investigate whether there are fibre type differences in the HSP response after BFRE, as eccentric exercise has been demonstrated to primarily damage and induce stress response in type 2 fibres (Fridén *et al.* 1983, Paulsen *et al.* 2009).

In the first days following stressful exercise, the expression of HSP27, αB-crystallin and HSP70 may increase (Paulsen *et al.* 2009). Curiously, it seems that HSP70 expression increases after both damaging and non-damaging muscle contractions (Morton *et al.* 2009b), whereas up-regulated expression of the small heat-shock proteins HSP27 and αB-crystallin seems to be more related to exercise-induced muscle damage (Morton *et al.* 2006, Paulsen *et al.* 2007, Jørgensen *et al.* 2013).

With the exception of the study of Wernbom *et al.* (2012), muscle damage at a cellular level (assessed by muscle biopsies) has until now not been investigated after BFRE. Interestingly, the cellular stress caused by low-load BFRE is probably very different from the stress studied in most other exercise models with high mechanical load. Consequently, it is of great interest to further explore the cellular stress of BFRE to increase our understanding of how this type of exercise affects the muscle cells, and further how this acute stress is translated into the training adaptations seen after repeated bouts. Furthermore, a recent case study, which showed that severe damage may occur in some individuals (Iversen & Røstad 2010), underlines the need for more detailed information regarding the stress response and safety aspects of this mode of exercise.

Therefore, the aim of this study was to investigate the myocellular response of HSP27, αB-crystallin and HSP70 after one bout of low-load BFRE. The effects of the low-load BFRE was compared with work-matched free-flow exercise (i.e. the same load and number of sets and repetitions). This study design allowed us to separate the effects of low-load resistance exercise with reduced blood flow from the effects of low-load resistance exercise alone.

The main hypothesis was that a larger HSP27, HSP70 and αB-crystallin translocation from the cytosolic to the cytoskeletal compartment would be observed after BFRE than after the free-flow exercise. Furthermore, we expected a larger increase in the levels of HSP70 in the BFRE leg than the free-flow leg. Finally, the HSP response was hypothesized to be larger in type 1 than in type 2 fibres.

Methods

Participants

Young and healthy males ($n = 7$) and females ($n = 2$) ($n = 9$; age 26 ± 3 years, height 177 ± 7 cm, body weight 79 ± 11 kg) were recruited. All participants were physically active, with varying degree of strength training experience. All participants gave written consent before the study and were informed about potential risks related to the experiment. The study was approved by the Regional Ethics Committee of

Southern Norway and was performed in accordance with the Helsinki Declaration.

Experimental design

The experimental design has previously been described in detail (Wernbom *et al.* 2012). In short, the participants were instructed not to do any strenuous activities during the last 72 h before the pre-testing or main experiment. One week before the experiment, one repetition maximum (1RM) of unilateral knee extension was tested in a knee extension machine (Gym2000, Vikersund, Norway). The main experiment was carried out with partial blood-flow restriction (BFR) induced by a 13.5-cm-wide pressure cuff connected to a tourniquet system (Zimmer A.T.S 2000, Zimmer Patient Care, Dover, OH, USA). The cuff was wrapped around the proximal part of the thigh and inflated to 90 (females) or 100 (males) mmHg. The rationale behind the choice of pressures is discussed in Wernbom *et al.* (2012, 2013). Following a light 5-min warm-up on a stationary bicycle (without BFR), five sets to failure at 30% of 1RM was performed during BFR on one randomly selected leg (dominant/non-dominant). Subsequently, with the same relative load, the corresponding number of repetitions was performed with the other leg without BFR. Forty-five seconds of rest was given between sets.

Strength testing

Isometric maximal voluntary contractions (MVC; 90° knee angle) were assessed for the knee extensors before and repeatedly after exercise in both legs, as described in detail in Wernbom *et al.* (2012).

Muscle sampling and homogenization

Muscle biopsies were sampled from the mid-portion of *m. vastus lateralis* at before exercise, 1, 24 and 48 h after exercise. The insertions of the repeated biopsies were placed 3 cm proximally from the previous biopsy. Under local anaesthesia (Xylocain adrenalin, 10 mg mL^{-1} + 5 μg mL^{-1}; AstraZeneca, London, UK), approx. 200 mg (2–3 × 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, before being weighed and quickly frozen in isopentane cooled on dry ice. Muscle biopsies for immunohistochemistry were mounted in a Tissue-Tek compound (Cat#4583; Sakura Finetek, Torrance, CA, USA) and quickly frozen in isopentane cooled on liquid nitrogen. All muscle samples were stored at −80 °C for later analyses.

About 50 mg of muscle tissue was homogenized and fractionated into cytosol, membrane and cytoskeletal fractions using a commercial fractionation kit (ProteoExtract Subcellular Proteome Extraction Kit, Cat#539790, Calbiochem, EMD Biosciences, Schwalbach, Germany) and according to the manufacturer's procedures. No parts were discarded in the fractionation process, and the purity of each fraction has earlier been tested in our laboratory showing both high levels of purity and no intercontamination. Protein concentrations were measured using 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, Cambridge, UK) and the provided software (Kim, ver. 5.45.0.1, Daniel Kittrich, Prague, Czech Republic).

Immunoblot

Homogenates were analysed using the western blot technique. Equal amount of protein was loaded per well (9-30 μg) and separated by 4–12% SDS-PAGE gels under denatured conditions for 35-45 min at 200 volts in cold MES running buffer (NuPAGE MES SDS running buffer; Life technologies, Invitrogen, Carlsbad, CA, USA). Samples analysed for HSP were transferred onto a PVDF membrane (iBlot Gel transfer stacks, Cat#IB4010; Life technologies, Invitrogen) for 6 min at 20 volts using iBlot dry western blot technique (iBlot dry blotting system, Cat#IB1001; Life technologies, Invitrogen). Samples analysed for desmin were transferred onto a PVDF membrane (Cat#162-0177; Bio-Rad), at 30 volts for 90 min in cold transfer buffer (NuPAGE transfer buffer, Cat#NP0006-1; Life technologies, Invitrogen). Membranes were blocked over night at 4 °C in a 5% fat-free skimmed milk and 0.05% TBS-t solution (TBS, Cat#170-6435; Bio-Rad; Tween-20, Cat#437082Q, VWR International, Radnor, PA, USA; Skim milk, Cat#1.15363; Merck, Germany). Blocked membranes were incubated with a primary monoclonal antibody against HSP70 (mouse anti-HSP70, Cat#ADI-SPA-810; Enzo Life Sciences, Farmingdale, NY, USA) or αB-crystallin (mouse anti-αB-crystallin, Cat#ADI-SPA-222, Enzo Life Sciences) diluted 1 : 4000, or a monoclonal antibody against desmin (mouse anti-desmin, Cat#Ab6322; Abcam, Cambridge, UK) diluted 1 : 3000; all at room temperature for 2 h. Membranes were then incubated with a secondary antibody (goat anti-mouse, Cat#31430; Thermo Scientific/Pierce Biotechnology, Rockford, IL, USA) diluted 1 : 30 000 at room temperature for 1 h. All antibodies were diluted in a 1% fat-free skimmed milk and 0.05% TBS-t solution at room temperature. Between stages, membranes were washed in 0.05% TBS-t. Bands were visualized using

a HRP detection system (Super Signal West Dura Extended Duration Substrate, Cat#34076 Thermo Scientific/Pierce Biotechnology). Chemiluminescence was measured using a CCD image sensor (Image Station 2000R; Eastman Kodak, Inc., Rochester, NY, USA), and band intensities were calculated with the Carestream molecular imaging software (ver. 5.0.6.20; Carestream Health, Inc., Rochester, NY, USA). All samples were analysed in duplicates, and mean values were used for statistical analyses.

ELISA

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 (25 ng per well; mouse anti-HSP27, Cat#ADI-SPA-800; Enzo Life Sciences) and a polyclonal detection antibody against HSP27 (rabbit anti-HSP27, Cat#ADI-SPA-803; Enzo Life Sciences) diluted 1 : 10 000. Horseradish peroxidase conjugate was used as secondary antibody, diluted 1 : 10 000 (Cat#RPN4301, Amersham Biosciences; GE Healthcare Life Sciences, Buckinghamshire, UK). The HSP27 assay was performed in high-binding polystyrene microplates (Cat#3590; Costar, Inc., Corning, NY, USA) using tetramethylbenzidine (TMB Solution, Cat#CL07, Calbiochem, EMD Biosciences, Germany) as substrate and 2 N sulphuric acid as stop solution. Recombinant HSP27 (Cat#SPP-715, Enzo Life Sciences) was used as standards (0.0975–25 ng mL^{-1}). All samples were diluted 1 : 300 (cytosolic fraction), 1 : 100 (membrane fraction) or 1 : 50 (cytoskeletal fraction) and analysed in triplicates (CV < 10%). The amount of HSP27 was determined using a filter photometer measuring optical density at 450 nm.

Histochemical staining

Eight-micrometre-thick cross sections were cut in a microtome at −20 °C (CM3050; Leica Biosystems GmbH, Wetzlar, Germany) and mounted on microscope slides (Superfrost Plus, Thermo Scientific, MA, USA), air-dried and stored at −80 °C. The muscle sections were blocked for 30 min with 1% BSA (bovine serum albumin; Cat#A4503, Sigma Life Science, St Louis, MO, USA) and 0.05% PBS-t solution (Cat#524650, Calbiochem, EMD Biosciences) before incubated with antibodies against HSP27 (Cat#ADI-SPA-803, Enzo Life Sciences), αB-crystallin, HSP70 or desmin diluted 1 : 200 in the blocking solution for 2 h at room temperature, followed by incubation with appropriate secondary antibodies (Alexa Fluor, Cat#A11005 or Cat#A11001; Life technologies, Invitrogen) for 30 min at room temperature. Muscle sections were visualized using a high-resolution camera (DP72, Olympus Corp., Tokyo, Japan) mounted on a microscope (BX61, Olympus Corp., Japan) with a fluorescence light source (X-Cite 120PCQ; EXFO Photonic Solutions Inc., Mississauga, ON, Canada). To compare staining intensities between muscle sections, camera and software settings were fixed. The muscle sections were then incubated with antibodies against myosin heavy chain type 1 (1:1000; BA-D5, gift from Prof. S. Schiaffino) and dystrophin (1:1000; Cat#ab15277, Abcam, Cambridge, UK) over night at 4 °C followed by incubation of an appropriate secondary antibody. Muscle sections were then covered with a coverslip and glued with ProLong Gold Antifade Reagent with DAPI (Cat#P36935, Invitrogen Molecular Probes, Eugene, OR, USA) and left to dry overnight at room temperature. Between stages, the sections were washed 3 × 5 min in 0.05% PBS-t solution. HSP staining intensity was analysed using ImageJ with Fiji image processing package (https://www.fiji.sc). All analysed fibres were quantified and related to their respectable fibre type. Each sample had the average of 61 (range: 8–169) type 1 fibres and 75 (range: 0–178) type 2 fibres analysed at each time point. An increase in staining intensity mainly represents an increase in total bound HSPs, or accumulation of bound HSPs in certain areas.

Glycogen content in fibres was visualized by periodic acid-Schiff (PAS) staining. The PAS staining was carried out according to the manufacturer's procedures (Cat#395-1, Cat#395-2, Cat#GHS-3; Sigma-Aldrich, Steinheim, Germany). To compare staining intensities between muscle sections, camera and software settings were fixed. For quantification of PAS staining intensity, each stain (haematoxylin and PAS) were separated using a colour deconvolution plugin (Landini, G; http://www.mecourse.com/landinig/software/cdeconv/cdeconv.html) for Fiji image processing package based on the algorithm by Ruifrok and Johnston (Ruifrok & Johnston 2001). Further analysis of staining intensity was analysed using ImageJ with Fiji image processing package. All analysed and quantified fibres were related to their respectable fibre type on a neighbouring muscle section. Each sample analysed with PAS staining had the average of 64 (range: 20–130) type 1 fibres and 96 (range: 25–163) type 2 fibres at each time point.

Statistics

All values are presented as means ± standard deviations. All data were tested for Gaussian distribution using a D'Agostino–Pearson omnibus normality test. A nonparametric Friedman test and Dunn's post hoc test with correction for multiplicity were used to

investigate changes over time for each leg. Differences between the BRFE leg and free-flow leg were analysed using a nonparametric Wilcoxon matched-pairs signed-rank test. A Kruskal–Wallis test with Dunn's multiple comparisons test was used to investigate changes in fibre distributions for glycogen staining intensity. Correlations were tested using the Pearson product-moment correlation coefficient test. Outliers were identified using the Grubbs' test. The level of significance was set to $P < 0.05$, and P values are reported as multiplicity-adjusted P values. GraphPad Prism 6 (San Diego, CA, USA, http://www.graphpad.com) was used for statistical analyses.

Results

The number of repetitions performed by the BFRE leg before failure decreased from 28 ± 5 in the first set to 10 ± 2 and 6 ± 1 in the second and fifth set, respectively (Wernbom *et al.* 2012), and the mean total number of repetitions was 56 ± 9.

Muscle force (MVC) was reduced in both legs immediately after exercise, but more in the BFRE leg (BFRE: 63 ± 15 vs. free flow: 23 ± 6%; $P < 0.001$). Normalization of muscle force was reached at 72 h post-exercise, but large individual differences were seen; one participant demonstrated a 49% force deficit in the BFRE leg at this time point. These data, as well as delayed onset muscle soreness, passive tension and low-frequency fatigue are previously published by Wernbom *et al.* (Wernbom *et al.* 2012).

HSP levels in cytosolic fraction

One hour post-exercise, the cytosolic HSP27 levels were more reduced in the BFRE leg than in the free-flow leg (−21 ± 19 vs. −2 ± 17%; $P = 0.039$; Fig. 1a). HSP27 levels in the BFRE leg were back to baseline 24 h post-exercise. No changes were observed for the free-flow leg at any time points. One hour post-exercise, the cytosolic αB-crystallin levels were significantly reduced in both legs compared with pre-exercise

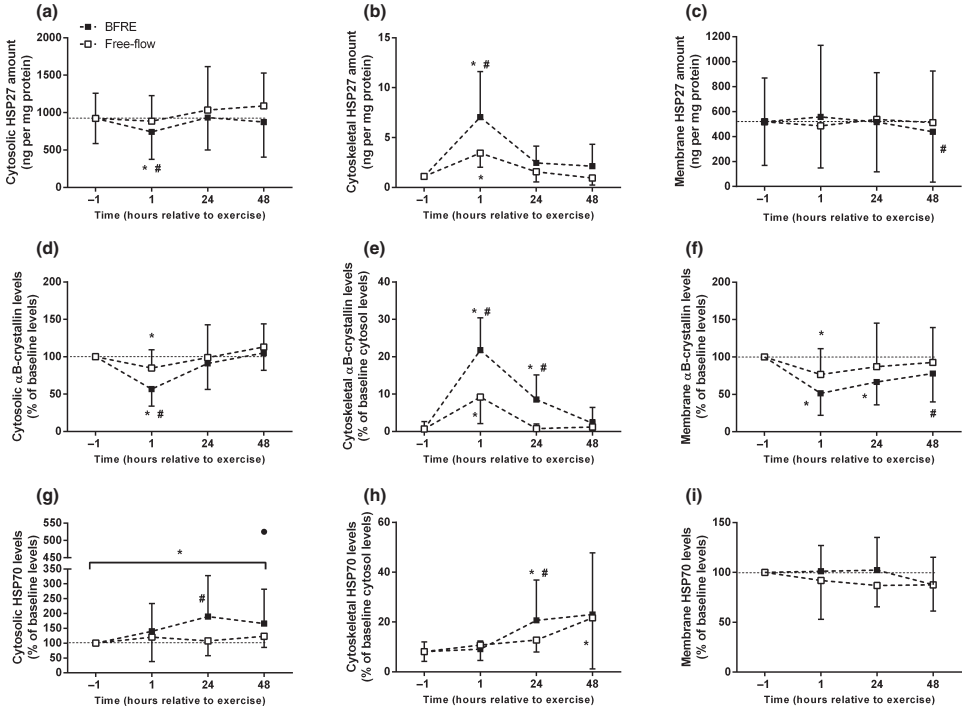

Figure 1 HSP27 content in the cytosolic (a), cytoskeletal (b) and membrane fraction (c), αB-crystallin levels in the cytosolic (d), cytoskeletal (e) and membrane fraction (f), and HSP70 levels in the cytosolic (g), cytoskeletal (h) and membrane fraction (i). Note that an outlier (data point shown as • in Fig. 1g) was removed from the cytosolic HSP70 dataset. HSP27 is expressed relative to total amount of protein (ng per mg total protein) and αB-crystallin, and HSP70 is expressed as relative values of pre-exercise/baseline (−1 h) values. Values are presented as mean±standard deviations. *Different compared with pre-exercise (−1 h) values ($P < 0.05$). #Differences between legs ($P < 0.05$).

values (−43 ± 23 vs. −15 ± 24%; $P = 0.003$ and $P = 0.014$, respectively; Fig. 1d). However, BFRE induced a larger decrease in the αB-crystallin levels 1 h post-exercise compared with free flow ($P = 0.009$; Fig. 1d). Twenty-four hours post-exercise, the levels of αB-crystallin were back to baseline values in both legs. For HSP70, no significant changes were seen in any of the exercised legs compared with pre-exercise values. However, when calculating area under the curve using the Trapezoid rule, a significant response was seen over the 48 h post-exercise in the BFRE leg compared to free-flow leg ($P = 0.039$; Fig. 1g). Note that one subject had extremely high values and defined as a significant outlier at this time point and removed from the data set (individual data point is shown in Figure 1g). Representative immunoblots for the cytosolic fractions are included as supplemental information.

HSP levels in the membrane fraction

αB-crystallin levels in the membrane fraction were reduced in both legs one hour post-exercise (−49 ± 29 vs. −23 ± 34%; $P < 0.001$ and $P = 0.014$, respectively; Fig. 1f). At 24 h post-exercise, αB-crystallin levels in the BFRE leg were still reduced compared with pre-exercise levels (−33 ± 29%; $P = 0.041$). At this time point, αB-crystallin levels in the free-flow leg were back to baseline. Although not significantly different from pre-exercise levels, αB-crystallin levels at 48 h were significantly lower in the BFRE leg compared with free-flow leg (22 ± 41% vs. 7 ± 50% respectively, $P = 0.035$). HSP27 remained unchanged from baseline in both legs at all time points (Fig. 1c), but at 48 h post-exercise, HSP27 levels were significantly lower in the BFRE leg compared to the free--flow leg (−9 ± 26 vs. 9 ± 22%, respectively; $P = 0.039$; Fig. 1c). HSP70 remained unchanged from baseline values in both legs, with no differences between legs (Fig. 1i). Representative immunoblots for the membrane fractions are included as supplemental information.

HSP levels in the cytoskeletal fraction

Compared with pre-exercise values, cytoskeletal HSP27 and αB-crystallin levels were increased in both legs 1 h post-exercise (BFRE: $P < 0.001$ and $P < 0.001$, respectively. Free flow: $P = 0.003$ and $P = 0.001$, respectively; Fig. 1b,e). The increases in both HSP27 and αB-crystallin were larger in the BFRE leg than free-flow leg at this time point (HSP27: 655 ± 439 vs. 310 ± 105%; $P = 0.008$. αB-crystallin: 22 ± 9 vs. 9 ± 7%; $P = 0.009$). In both legs, HSP27 levels were back to baseline 24 h post-exercise. In the BFRE leg, levels of αB-crystallin remained increased 24 h post-exercise compared with pre-exercise values ($P = 0.041$) and higher than in free-flow leg ($P = 0.014$; Fig. 1e), but returned to baseline 48 h post-exercise. The αB-crystallin levels in the free-flow leg were back to baseline 24 h post-exercise. For HSP70, higher protein levels in the cytoskeletal fraction were found at 24 h post-exercise in the BFRE leg ($P = 0.024$; Fig. 1h) and at 48 h post-exercise in the free-flow leg compared with pre-exercise values ($P = 0.006$; Fig. 1h). At 24 h post-exercise, higher HSP70 levels were found in the BFRE leg than free-flow leg (21 ± 16 vs. 13 ± 5%. $P = 0.042$; Fig. 1h). Representative immunoblots for the cytoskeletal fractions are included as supplemental information.

Fibre-specific changes in the HSP response

Type 1 fibres had significantly higher HSP70 and αB-crystallin staining intensity baseline than type 2 fibres ($P = 0.008$ and $P = 0.004$, respectively). Relative to pre-exercise values, a significant increase in HSP70 staining intensity was seen in type 1 fibres at 24 and 48 h post-exercise in the BFRE leg (171 ± 116 and 86 ± 35%, respectively; $P = 0.002$ and $P = 0.011$; Fig. 2a). These changes in staining intensity were significantly larger than in the free-flow leg ($P = 0.016$ and $P = 0.008$, respectively; Fig. 2a). No increase was observed in type 2 fibres, but a tendency ($P = 0.075$) to differences in staining intensity between the BFRE and free-flow leg was observed at 24 h post-exercise (Fig. 2b). αB-crystallin staining intensity was increased in both legs 1 h after exercise (Fig. 3). The increased αB-crystallin staining intensity was manifested in type 1 fibres in both legs 1 h post-exercise (BFRE: $P = 0.02$. Free flow: $P = 0.012$; Fig. 2c,d), but there was no change in type 2 fibres. At 48 h, αB-crystallin staining intensity was significantly reduced in type 2 fibres in the free-flow leg ($P = 0{,}019$; Fig. 2d), but was not different from the BFRE leg. The staining pattern for αB-crystallin and HSP70 showed generally an evenly stain spread out across the fibres (Fig. 4c). A punctate, granular staining pattern for αB-crystallin, as frequently observed after eccentric exercise, was only observed in some fibres in a few participants (Fig. 4a). Low staining and no change over time were observed for HSP27 in both fibre types.

Desmin

Desmin levels measured by immunoblots remained unchanged in both legs relative to pre-exercise values (Fig. 5). Immunohistochemical staining against desmin did not show any overt changes in staining intensity at any time point or between legs.

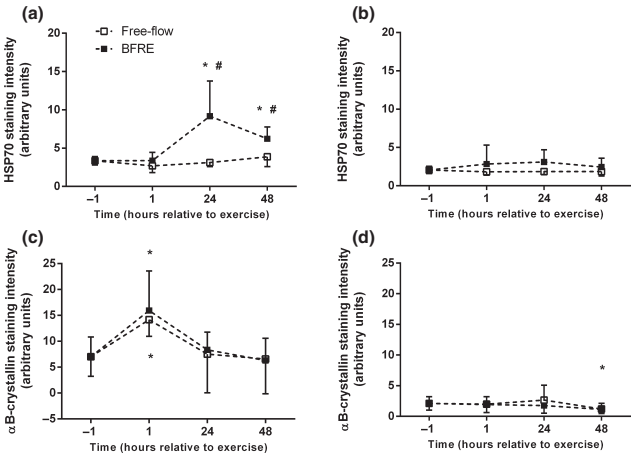

Figure 2 HSP70 staining intensity in type 1 (a) and type 2 fibres (b) and αB-crystallin staining intensity in type 1 (c) and type 2 fibres (d). Values are presented as mean ± standard deviations. *Different compared with pre-exercise/baseline (−1 h) values ($P < 0.05$). #Differences between legs ($P < 0.05$).

Glycogen staining

Relative to pre-exercise values, glycogen content in type 1 fibres was reduced in both legs at 1 and 24 h ($P < 0.001$; Fig. 6). At 48 h, only the BFRE leg showed reduced glycogen content relative to pre-exercise ($P < 0.001$) and it was significantly lower than in the free-flow leg ($P < 0.001$). Type 2 fibres in the BFRE leg showed reduced glycogen content 1 and 24 h post-exercise relative to pre-exercise ($P < 0.001$; Fig. 6), while in the free-flow leg, only reduced glycogen content was observed at 24 h post-exercise ($P = 0.006$). Compared with the free-flow leg, BFRE displayed less glycogen content post-exercise in both type 1 and 2 fibres at all time points ($P < 0.001$; Fig. 6a–f). The same fibres that had reduced glycogen content 48 h post-exercise were observed to display high staining for HSP70 (Fig. 7).

Correlations

The reductions in cytosolic levels of αB-crystallin were correlated with muscle force deficits at 24 h post-exercise, $r = 0.75$ ($P = 0.02$) for the BFRE leg and $r = 0.61$ ($P = 0.007$) for both legs combined. Furthermore, increases in cytoskeletal HSP27 in the BFRE leg correlated with reductions in force-generating capacity at 24 h, $r = 0.87$ ($P = 0.002$).

Discussion

The main finding in the present study was that exercising with low loads (30% of 1RM) induced a HSP response in the exercised muscle which was more pronounced in type 1 than in type 2 fibres and was aggravated by blood-flow restriction. The most rapid response was seen for the small HSPs; HSP27 and αB-crystallin, which accumulated in cytoskeletal structures 1 h post-exercise. HSP70 displayed a more delayed response, and in contrast to the small HSPs, the total amount of HSP70 was increased during the first 48 h post-exercise, but only significantly in the blood-flow-restricted leg. The delayed HSP70 response in the affected type 1 fibres was paralleled by prolonged glycogen depletion in these fibres (discussed further below).

The small HSPs response

The small HSPs, HSP27 and αB-crystallin, have been suggested to have special cytoprotective properties as they translocate and accumulate in damaged and/or stressed myofibrillar structures during ischaemia or high-force exercise (Armstrong *et al.* 1999, Yoshida *et al.* 1999, Koh 2002, Golenhofen *et al.* 2004, Koh & Escobedo 2004, Paulsen *et al.* 2009). The biological role of small HSPs is to act as protein chaperones that interact with partly and completely unfolded proteins (Mymrikov *et al.* 2011). As hypothesized in the exercised muscles, both HSP27 and αB-crystallin translocated from the cytosolic to the cytoskeletal fractions acutely after exercise (Fig. 1a–b, d and e). Hence, the direct interaction causing the observed translocation and binding to the stressed structures seems to be exposure of hydrophilic binding sites during denaturation. Consequently, the small HSPs accumulate in stressed and damaged areas within the muscle cell even after low-load resistance exercise. Interestingly, the translocation was significantly larger in the BFRE leg than in the free-flow leg, which suggests that BFR induces stress superimposed on the exercise stress (both legs performed similar work).

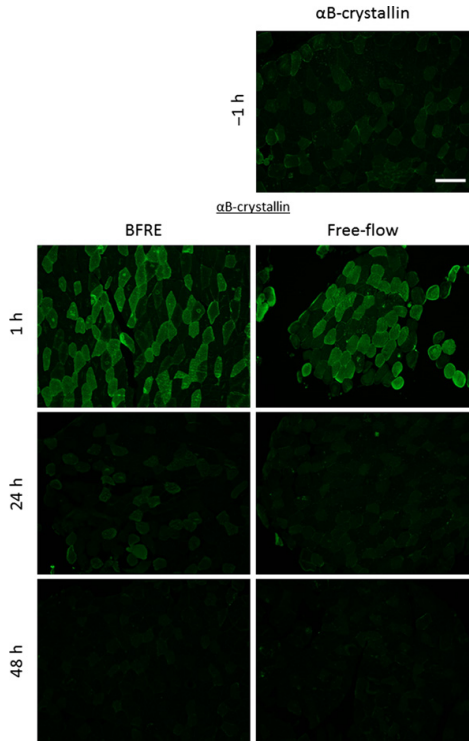

Figure 3 Representative images of muscle cross sections stained for αB-crystallin from the BFRE (left panel) and free-flow (right panel) leg for all time points. The αB-crystallin staining shows increased staining 1 h after exercise in both legs and decreased staining at 24 and 48 h post-exercise. Scale bar = 200 μm (Pre-exercise/−1 h image).

In a study by Golenhofen *et al.* (2004), rat skeletal muscles (soleus and EDL) were exposed to ischaemic conditions, which induced a rapid (<30 min) accumulation of both HSP25 (homologous to human HSP27) and αB-crystallin in cytoskeletal structures (especially in the I-band area). This suggests that the reduced oxygen delivery to the exercising muscles during BFR, in which ischaemic conditions occur, might be responsible for the increased translocation of small HSPs to cytoskeletal structures. The apparently stable desmin levels observed in our study (Fig. 5) indicate, however, that brief ischaemic stress does not cause gross sarcomeric/myofibrillar disruptions and/or degradation, as seen after eccentric exercise (Barash *et al.* 2002). Moreover, we rarely observed granular, punctuated staining pattern of the small HSPs on cross sections, which has been a hallmark for myofibrillar disruption after eccentric and heavy load resistance exercise (Paulsen *et al.* 2007, 2009, Folkesson *et al.* 2008). Indeed, Paulsen *et al.* (2009) demonstrated that the immunohistochemical staining pattern of the small HSPs on cross sections identifies areas of myofibrillar disruptions, as evaluated by immunostainings of whole fibre preparation (confocal microscopy) and by immunogold HSP labelling of disrupted sarcomeres/myofibrils (electron microscopy). As we in the present study applied the same staining protocols and the same antibodies as previously used (Paulsen *et al.* 2007, 2009), we believe that translocation to the cytoskeleton is due to a type of stress/damage that does not include disruptions of the myofibrillar structures, for example ischaemic stress. Thus, the exact type of cellular stress and damage caused by BFRE is not known, but seems clearly distinguishable from eccentric exercise-induced damage.

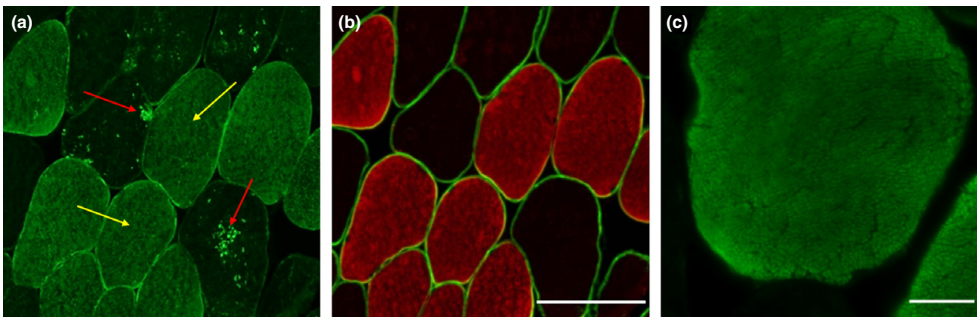

Figure 4 Muscle cross section from 1 h post-exercise stained for αB-crystallin showing punctate, granular staining (red arrows) and fibres with even staining (yellow arrows) (a) and staining against myosin heavy chain 1 (red) and dystrophin (green) (b), scale bar = 100 μm. High magnification of cross section showing an evenly spread staining pattern for αB-crystallin across the fibre (c), scale bar = 20 μm. Note that all fibres with increased even staining intensity are type 1 fibres.

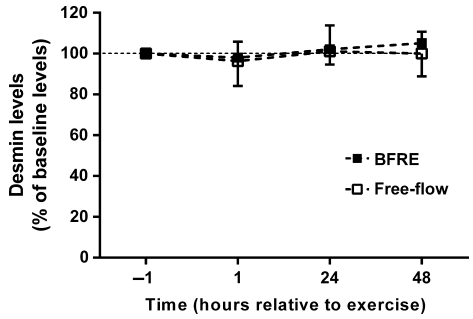

Figure 5 Desmin levels in both legs relative to baseline levels. Desmin levels are expressed as relative change (%) to pre-exercise/baseline (−1 h) values. No changes were seen in desmin levels at any time point or between legs. Values are presented as mean ± standard deviations.

The HSP70 response

Compared with HSP27 and αB-crystallin, HSP70 exhibited a slower response after BFRE and there were no apparent translocation to the cytoskeletal fraction 1 h post-exercise (Fig. 1g,h). The HSP70 response in the cytosolic fraction of the BFRE leg, measured over 48 h, showed larger increase compared with the free-flow leg. This is most likely due to the ischaemic conditions superimposed on the exercised muscles in the BFRE leg. According to previous studies, significantly increased HSP70 levels have been found 2–6 days post-exercise after aerobic exercise in a single-legged cycle ergometer (Khassaf *et al.* 2001, 2003, Jackson *et al.* 2004) or treadmill running (Morton *et al.* 2006), but also 1–7 days after muscle-damaging eccentric exercise (Paulsen *et al.* 2009, 2007; Thompson *et al.* 2002, 2003). Collectively, the typically delayed and prolonged HSP70 response indicates that HSP70 is involved in the recovery and/or the adaptation processes after stressful exercise.

Increased ischaemic stress by BFRE seems to accelerate the synthesis of HSP70 – particularly in the type 1 fibres – which may improve the fibres stress tolerance. This suggestion is supported by studies demonstrating improved recovery from eccentric contractions in mice and rats that overexpress HSP70 (McArdle *et al.* 2004) or have undergone heat shock pre-treatment (Touchberry *et al.* 2012). Furthermore, rat muscles in which the HSP70 levels are increased by heat stress suffered less reperfusion damage than unconditioned muscles (Lepore *et al.* 2000, Baumeister *et al.* 2004). Similarly, in mouse myoblasts (C_2C_{12}), less cell damage was induced by chemical agents when the myoblasts were exposed to heat pre-conditioning (Maglara *et al.* 2003). Importantly, this was the first time our participants performed BFRE, and as a

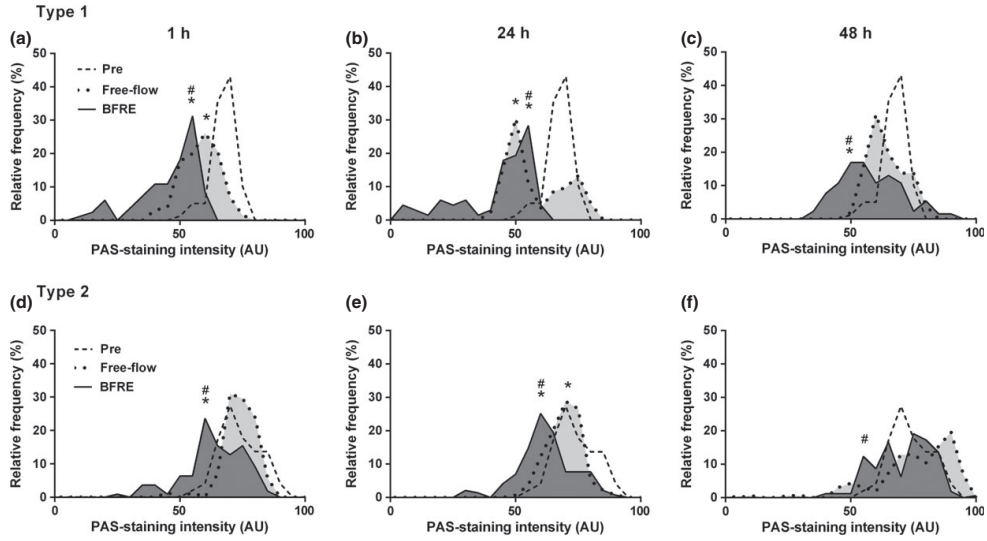

Figure 6 Relative frequency distributions (%; intervals of 5) of PAS staining intensity in type 1 (a–c) and type 2 fibres (d–f), for BFRE leg (dark grey-solid lines) and free-flow leg (light grey-dotted lines). Distribution from pre-exercise sample is indicated with dashed lines. Note that values are normalized to equal low/no PAS staining intensity (0) and high PAS staining intensity (250). *Different compared with pre-exercise/baseline (−1 h) values ($P < 0.05$). #Differences between legs ($P < 0.05$).

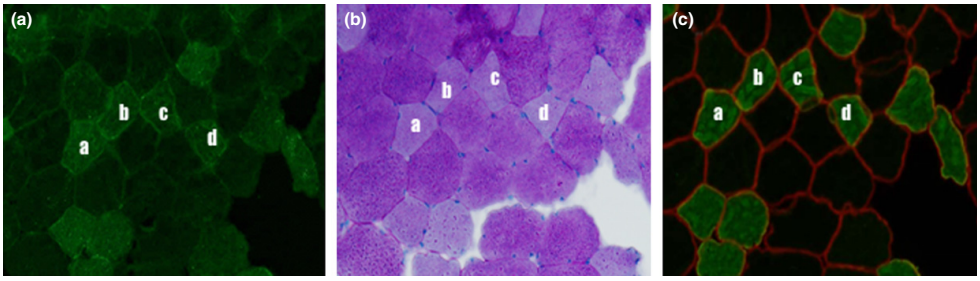

Figure 7 Neighbouring muscle cross sections stained with HSP70 (a), PAS staining (b) and fibre type 1 (MHC 1); (c), showing co-localization of increased HSP70 immunoreactivity with glycogen depletion in type 1 fibres 48 h after exercise.

consequence of increased HSP70 levels, and other possible adaptations to this first bout of BFRE, it is reasonable to suggest that less stress and damage would be induced in a repeated bout some days later. Consequently, the magnitude of the HSP responses observed in this study is only representative for participants unaccustomed to BFRE.

Preferential response in type 1 fibres. Immunohistochemical stainings showed higher baseline αB-crystallin and HSP70 staining intensity in type 1 compared with type 2 fibres. This verifies previous findings (Paulsen *et al.* 2009, Larkins *et al.* 2012), but not all previous findings (Tupling *et al.* 2007, Folkesson *et al.* 2013). In agreement with the low amount of HSP27 we found in the cytoskeletal fraction with ELISA, no (or low) staining intensity was observed for HSP27, which indicates low amounts of bound HSP27. After exercise, the staining intensity for αB-crystallin was acutely increased only in type 1 fibres, but in both legs. This finding is in line with the increased binding to cytoskeletal structures observed with immunoblots and gives additional information on the more specific location in type 1 fibres. HSP70 staining intensity was also increased only in type 1 fibres, but this was at later time points and only in the BFRE leg (Fig. 2a). Increased staining intensity indicates increased HSP70 binding to structural proteins. In agreement with our immunoblot data, increased staining intensity is a result of increased bound HSP70 to cytoskeletal structures.

The overt increase in HSP70 staining in type 1 fibres is in agreement with observations after submaximal isometric contractions, as reported by Tupling *et al.* (2007). This is an interesting finding, as it has earlier been demonstrated that both type 1 and 2 fibres are recruited during BFRE performed to failure (Krustrup *et al.* 2009). The preferential increase in HSP70 staining in type 1 fibres indicates that these fibres are highly affected by BFRE, which is in contrast to findings after eccentric exercise with predominantly increased HSP staining in type 2 fibres (Paulsen *et al.* 2009). However, as type 2 fibres seem to be recruited later than type 1 fibres with low-load ischaemic exercise (Moritani *et al.* 1992), type 1 fibres was exposed to considerably longer durations of metabolic stress with our exercise protocol.

The exercise protocol reduced glycogen content, especially in type 1 fibres. Moreover, the reduction was more prominent in the BFRE leg than free-flow leg. Interestingly, fibres that were glycogen depleted (or had low glycogen levels) also displayed a strong staining intensity against HSP70 48 h after exercise. This may indicate that the increased HSP70 levels were linked to reduced energy availability in the fibres. In fact, HSPs have been shown to increase when glucose availability is low (Febbraio *et al.* 2002). In contrast to our findings, Tupling *et al.* (2007) reported preferentially glycogen depletion in type 2 fibres concomitantly with increased HSP70 content in type 1 fibres.

Intriguingly, the glycogen content in type 1 fibres appeared to stay low for at least 48 h in the BFRE leg. This suggests that BFRE somehow has impaired the glucose uptake, as seen after eccentric exercise (Asp *et al.* 1995, 1996). It could therefore be a possibility that the stress induced by BFRE negatively affected the GLUT-4 transporters ability to translocate to the sarcolemma, either by damaging sarcolemma, as seen after BFRE (Wernbom *et al.* 2012), or by impairing insulin signalling.

Altogether, the fibre-specific HSP responses (αB-crystallin and HSP70) and the acute and prolonged glycogen depletion strongly indicate that type 1 fibres were more stressed than type 2 fibres during low-load BFRE. Because only two female participants were included in this study, it was not possible to statistically test for sex differences in the HSP response. However, the HSP response in the two female participants was of similar magnitude and direction as for

the males. In support of this observation, Gjøvaag & Dahl (2006) did not observe any sex differences in HSP27 or HSP70 after strength training. Still, certain sex differences may exist as Morton *et al.* (2009a) reported that females did not display the same changes in αB-crystallin and HSP60 as males after endurance training.

Functional implications. Linking the cellular changes observed in this study (biopsies of *m. vastus lateralis*) to changes in muscle function of the knee extensors is inherently difficult. Nevertheless, maximal force was compromised in both the BFRE leg and free-flow leg, and numeric correlations were found between changes (subcellular movement) in the small HSPs, HSP27 and αB-crystallin, and changes in muscle force 24 h post-exercise. Such observations correspond with our previous findings after muscle-damaging eccentric exercise (Paulsen *et al.* 2009), and they imply that the HSP response reflects the cellular stress that in turn is related to impairment in muscle function. Studies by others support this notion (Noble *et al.* 2008), and collectively this underpins the importance to further investigate the HSP response to exercise and its relations to changes in muscle function, as such knowledge could have both clinical and sport-related implications. Finally, it is important to emphasize that our participants were not familiar with BFRE. Consequently, our results only reflects the stress from the first exercise bout with BFRE and logically this kind of exercise will be less stressful, better tolerated, in the succeeding bouts in a training programme.

Conclusion

In the present study, we explored for the first time the myocellular stress response to blood-flow restriction during exercise (BFRE). Blood-flow restriction clearly added cellular stress to the exercise protocol. BFRE induced a rapid translocation of HSP27 and αB-crystallin from the cytosolic and membrane to the cytoskeletal cell compartments, indicating that cytoskeletal proteins are stressed during this type of exercise. However, by means of analyses by immunohistochemistry and changes in desmin levels, we did not observe signs of overt myofibrillar disruptions and damage, as typically found after eccentric exercise, and the stress response was mainly located to type 1 fibres. Furthermore, the content of HSP70 increased after BFRE, especially in type 1 fibres, and this might be an adaptation increasing the tolerance to similar exercise. Finally, reduced glycogen content at 48 h after exercise indicates some impairment of glucose uptake and storage after BFRE. This observation was restricted to type 1 fibres and indicates that not only cytoskeletal proteins but also membrane and cytosolic proteins might be affected when participants are unaccustomed to this kind of exercise.

Conflict of interest

There is no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative immunoblots for αB-crystallin and HSP70.

Paper 2

Cumming, K. T., S., Ellefsen, S. B. R. Rønnestad, and T. Raastad. Acute and long-term effects of low load blood flow restricted resistance training on heat shock proteins and endogenous antioxidant systems in skeletal muscles of untrained females.

Paper 3

Cumming, K. T., T. Raastad, G. Holden, N. E. Bastani, D. Schneeberger, M. P. Paronetto, N. Mercatelli, H. N. Østgaard, I. Ugelstad, D. Caporossi, R. Blomhoff, and G. Paulsen. (2014). Effects of vitamin C and E supplementation on endogenous antioxidant systems and heat shock proteins in response to endurance training. Physiol Rep 2(9):e12142.

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ORIGINAL RESEARCH

Effects of vitamin C and E supplementation on endogenous antioxidant systems and heat shock proteins in response to endurance training

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Abstract

Reactive oxygen and nitrogen species are important signal molecules for adaptations to training. Due to the antioxidant properties of vitamin C and E, supplementation has been shown to blunt adaptations to endurance training. In this study, we investigated the effects of vitamin C and E supplementation and endurance training on adaptations in endogenous antioxidants and heat shock proteins (HSP). Thirty seven males and females were randomly assigned to receive Vitamin C and E (C + E; C: 1000 mg, E: 235 mg daily) or placebo (PLA), and underwent endurance training for 11 weeks. After 5 weeks, a subgroup conducted a high intensity interval session to investigate acute stress responses. Muscle and blood samples were obtained to investigate changes in proteins and mRNA related to the antioxidant and HSP system. The acute response to the interval session revealed no effects of C + E supplementation on NFκB activation. However, higher stress responses to exercise in C + E group was indicated by larger translocation of HSPs and a more pronounced gene expression compared to PLA. Eleven weeks of endurance training decreased muscle GPx1, HSP27 and αB-crystallin, while mnSOD, HSP70 and GSH remained unchanged, with no influence of supplementation. Plasma GSH increased in both groups, while uric acid decreased in the C + E group only. Our results showed that C + E did not affect long-term training adaptations in the antioxidant- and HSP systems. However, the greater stress responses to exercise in the C + E group might indicate that long-term adaptations occurs through different mechanisms in the two groups.

Introduction

Metabolic demands during exercise are associated with augmented formation of reactive oxygen and nitrogen species (RONS; Davies et al. 1982). High levels of RONS may cause oxidative damage to proteins, nucleotides, and lipids; and ultimately RONS can impair cell functions or induce necrosis (Peternelj and Coombes 2011). To counteract these apparently negative effects of high intensity exercise, it is widely common among athletes to use dietary vitamin/antioxidant supplements (Sobal and Marquart 1994). However, although increased RONS production during exercise has potential negative effects, transient increases in RONS seem to be a trigger for many exercise-induced adaptations in skeletal muscle (Powers et al. 2010). Indeed, recent studies indicate that antioxidant

supplementation may blunt mitochondrial biogenesis induced by endurance training in both animal and human models (Gomez-Cabrera et al. 2008; Paulsen et al. 2014).

Improved aerobic capacity appear to occur concomitantly with up-regulation of endogenous antioxidant systems (Powers and Jackson 2008) and heat shock proteins (HSP) in skeletal muscle (Morton et al. 2009b). While the main role of the antioxidant enzymes is to decrease oxidation and prevent oxidative damage, HSPs can prevent and reverse damage to proteins. Intriguingly, the HSPs cooperate with the antioxidant systems, and collectively they have essential roles in cell homeostasis. Up-regulation of these proteins is, therefore, important adaptations for increased protection and recovery capacity in face of cellular stress and damage induced by high-intensity exercise (Powers and Jackson 2008; Morton et al. 2009b).

Like in mitochondrial biogenesis, alterations in redox status modulate the training-induced adaptations in antioxidant systems and HSP systems (Gomez-Cabrera et al. 2005; Fittipaldi et al. 2014). A potential target for antioxidant supplementation might be the stress- and RONS sensitive NFκB p65 pathway (nuclear factor kappa B p65/RelA), which can activate gene expression of the antioxidant enzymes manganese superoxide dismutase (mnSOD) and glutathione peroxidase 1 (GPx1; Wan et al. 1994; Zhou et al. 2001) and HSP70 (Tranter et al. 2010; Sasi et al. 2014). Furthermore, HSP gene expression seems to also be activated via HSF1 with increased RONS levels (Jacquier-Sarlin and Polla 1996). This indicates that antioxidants have the potential to modulate the expression of stress-related proteins by decreasing RONS levels.

In agreement with this assumption, antioxidant supplements appear to inhibit adaptations in both antioxidant enzymes and the heat shock proteins (Peternelj and Coombes 2011), although the literature is equivocal. Some authors claim that antioxidant supplementation blunt resting mRNA expression of *SOD2* (mnSOD), *GPx1* and *HSPA4* (HSP70; Gomez-Cabrera et al. 2005, 2008; Fischer et al. 2006; Ristow et al. 2009), while others document no interference on protein levels (Jackson et al. 2004; Fischer et al. 2006; Yfanti et al. 2011, 2012; Gliemann et al. 2013). Furthermore, antioxidants have been shown to abolish the acute exercise-induced increases in HSP60 and HSP70 protein levels (Khassaf et al. 2003; Jackson et al. 2004).

In addition to the intra-cellular systems in muscle fibers, the plasma offers a variety of different antioxidant systems, like glutathione (GSH) and uric acid. Intriguingly, vitamin C supplementation effectively decrease uric acid levels (Juraschek et al. 2011). Research on the combined effects of antioxidant supplements and endurance training on GSH and uric acid levels in healthy individuals is, however, lacking, and further studies are needed to shed light over a possible interaction effect.

Due to the conflicting data and variations in methods used in previous studies, further investigation is needed to understand how antioxidant supplementation potentially affects both the acute stress response and the training adaptations in antioxidant systems and the heat shock proteins. This knowledge could have both clinical and sport-related interest, as these proteins are important to prevent damage and optimal recovery processes.

Consequently, the aim of this study was to investigate the effects of vitamin C and E supplementation on adaptations in the endogenous antioxidant systems and heat shock proteins after 11 weeks of endurance training. Further, we wanted to investigate how the supplements would affect acute stress responses to a high-intensity interval session midway into the training intervention. We hypothesized that vitamin C and E supplementation would decrease the exercise-induced increase of RONS, and thereby blunt the acute NFκB activation and the concomitant increase in gene expression of endogenous antioxidants (*SOD2*, *GPx1*) and heat shock proteins (*HSPA4* and *CRYAB*) in muscle (*m. vastus lateralis*) after a session of high-intensity interval running. Due to the accumulation of stress over time, muscle heat shock protein (αB-crystallin, HSP27 and HSP70) levels were expected to increase in both groups, but to a lesser degree with vitamin C and E supplementation. Furthermore, we hypothesized that vitamin C and E supplementation over 11 weeks of training would blunt the training-induced upregulation of endogenous antioxidant systems (mnSOD, GPx1 and GSH) in the muscle and plasma (GSH, uric acid, and total antioxidant capacity).

Methods

Participants

Nineteen male and 18 female participants completed the study. All participants were physical active, conducting regularly endurance training (1–4 times per week) before starting the study. Physical activity prior to the study was reported with a questionnaire by the participants. 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 Southern Norway and was performed in accordance with the Helsinki Declaration.

Supplements

The vitamin C and E and placebo pills were produced under Good Manufacturing Practice (GMP) requirements at Petefa AB (Västra Frölunda, Sweden). Each vitamin pill contained 250 mg of ascorbic acid and 58.5 mg DL-α-tocopherol acetate. The placebo pills had the same shape and appearance as the vitamin pills. All supplements were stored in unlabeled boxes.

The participants ingested two pills (500 mg of vitamin C and 117 mg vitamin E) 1–3 h before every training session and two pills in the hour after training. On nontraining days, the participants ingested two pills in the morning and two pills in the evening. The intake of pills was confirmed with an online training diary. Thus, daily dosage was 1000 mg of vitamin C and 235 mg vitamin E. Compliance to the supplements were 97 ± 5% (Paulsen et al. 2014). The total supplemental dosage of vitamin C was ~13 times higher than the recommended daily dietary allowance in the Nordic countries, and ~23 times higher for vitamin E.

Besides the supplementation given in the study, the participants were informed not to take any form of nutritional supplements. They were told not to drink more than two glasses of juice and four cups of coffee or tea per day. Juices especially rich in antioxidants, such as grape juice, were to be avoided.

Training

The training protocol used in the present study has been described in detail previously (Paulsen et al. 2014). In short, after a baseline VO_{2max} test, the participants were randomly assigned to receive vitamin C and E or a placebo supplement. The training consisted of three blocks over 11 weeks, where three running sessions were conducted for the first three weeks. Thereafter, the participants increased to four sessions per week. The exercise durations increased linearly in each exercise block. The training sessions were a mix of low- (72–82% of HR_{max}, 60 min), moderate- (82–87% of HR_{max}, 30 min), and high intensity (>90% of HR_{max}, intervals 4–6 × 4–6 min). Exercise intensity was calculated from maximal heart rate and the participants' perceived exertion using the Borg Scale (RPE). During each training session, heart rate and exercise intensity was monitored and logged using a heart frequency monitor (RS800CX; Polar Electro Oy, Tempere, Finland), and type of exercise and session RPE were logged using an online training log after each training session. For variation and motivation, participants were allowed to do alternative exercise forms (e.g., cycling, cross-country skiing) once per week, including a maximum of two bouts of other activities in addition to the planned training sessions.

Acute exercise bout

After 5 weeks of training, a subsample of participants (n = 16; five males, 11 females; age 23 ± 2 years, height 172 ± 8 cm, body weight 65 ± 10 kg VO_{2max} 59 ± 8 mL/kg/min) volunteered to complete an exercise bout of 4 × 4 min high-intensity interval training (>90% of maximal heart rate [HR_{max}]). The exercise session was performed on a treadmill (ELG 90/200 Sport; Woodway GmbH, Weil am Rhein, Germany). Exercise intensity was monitored using a heart rate monitor. VO_2 was measured during the last 1.5 min of each 4-min interval using a mixing chamber (Oxycon Pro; Erich Jaeger GmbH, Hoechberg, Germany), and blood lactate was measured (YSI 150 Sport Lactate Analyzer; YSI Inc., Yellow Springs, OH) immediately after each interval using a finger stick. The participants ingested the supplements 1–3 h before and immediately after the exercise bout. Blood samples were collected before exercise and 10, 90, and 180 min postexercise. Muscle biopsies were collected pre-exercise and 90 min postexercise.

Muscle tissue sampling and handling

Muscle biopsies from the mid-portion of the right *m. vastus lateralis* were collected under local anesthesia (Xylocain adrenalin, 10 mg/mL + 5 μg/mL; AstraZeneca PLC, London, UK) before and after the training intervention as previously described (Cumming et al. 2014). The post-training insertion was proximally located to the pre-training site (approximately 3 cm). For the participants that also took part in the mid-way, acute exercise bout, biopsies were collected from the left thigh. Tissue intended for mRNA analyses were placed in RNAlater (Cat#AM7020, Ambion; Life technologies, Carlsbad, CA). All muscle samples were stored at −80°C for later analyses.

Protein immunoblot and ELISA

Muscle tissue was homogenized and fractionated into cytosol-, membrane-, nuclear- and cytoskeletal fractions, using a commercial fractionation kit according to the manufacturer's procedures (ProteoExtract Subcellular Proteome Extraction Kit, Cat#539790, Calbiochem; EMD Biosciences, Schwalbach, Germany). Protein concentrations were assessed with a commercial kit (Bio-Rad DC protein micro-plate assay, Cat#0113, Cat#0114, Cat#0115; Bio-Rad, Hercules, CA), a filter photometer (Expert 96; ASYS Hitech, Cambridge, UK) and the provided software (Kim, ver.5.45.0.1, Daniel Kittrich, Prague, Czech Republic).

Proteins extracted from muscle samples were analyzed by western blotting as previously described (Cumming et al. 2014). Briefly, equal amounts of protein were

loaded per well (7–28 μg) and separated in 4–12% SDS-PAGE gels (Cat#NP0321, NuPAGE; Invitrogen, Life technologies) under denatured conditions. Proteins were transferred to PVDF-membranes (Cat#162-0177; Bio-Rad), before being blocked in a 5% fat-free skimmed milk and 0.05% TBS-t solution (TBS, Cat#170-6435, Bio-Rad; Tween 20, Cat#437082Q, VWR International, Radnor, PA; Skim milk, Cat#1.15363; Merck, Darmstadt, Germany). Antibodies against GPx1 (rabbit-anti GPx1, Cat#ab22604; Abcam, Cambridge, UK), mnSOD (mouse-anti mnSOD, Cat#ab16956; Abcam), NFκB p65 (anti-rabbit NFκB p65, Cat#ab7970; Abcam), IκBα (anti-rabbit IκBα, Cat#ab32518; Abcam), HSP70 (mouse-anti HSP70, Cat#ADI-SPA-810; Enzo Life Sciences, Farmingdale, NY) or αB-crystallin (mouse-anti αB-crystallin, Cat#ADI-SPA-222; Enzo Life Sciences), and appropriate secondary antibodies (goat anti-mouse, Cat#31430; Thermo Scientific, Rockford, IL, or Goat anti-rabbit, Cat#7074; Cell Signaling Technology, Danvers, MA) were used. Bands were visualized using a HRP-detection system (Super Signal West Dura Extended Duration Substrate, Cat#34076; Thermo Scientific). Chemiluminescence was measured using a CCD image sensor (Image Station 2000R or Image Station 4000R; Eastman Kodak Inc., Rochester, NY) and band intensities were calculated with the Carestream molecular imaging software (Carestream Health Inc., Rochester, NY). All samples were run as duplicates and mean values were used for statistical analyses.

GPx1, IκBα, HSP70 was analyzed using the cytosolic fraction, mnSOD using the membrane- and cytosolic fraction (results represents the combined results from both fractions), αB-crystallin using the cytosolic and cytoskeletal fraction (only cytosolic fraction for the pre-post results) and NFκB p65 using the cytosolic- and nuclear fraction.

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

RT-qPCR

Total RNA was extracted from muscle biopsies (n = 10) from the acute study by homogenization of samples in TRIzol reagent (Cat# 15596; Invitrogen, Life Technologies) according to the manufacturer's procedures. DNase I digestion was performed using RNase free-DNase from Qiagen (Cat#79254; Qiagen Inc., Germantown, MD) in order to prevent genomic DNA contamination. Quantitative-PCR (q-PCR) analysis was performed in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). qPCR reactions were performed using Power SYBR Green RNA-to-Ct ™ 1-step Kit (Cat# 4389986; Applied Biosystems) supplemented with forward and reverse primer in a total volume of 20 μL. The reverse transcription step was performed co-incubating the PCR primers with 6 ng of RNA and the MultiScribe™ Reverse Transcriptase (Cat#4311235; Invitrogen, Life Technologies) at 48°C for 30 min in the presence of RNase Inhibitor. Thermocycling conditions were according to the recommendations of the manufacturer. *Ct* values for gene expression were calculated according to the comparative *Ct* method (Pfaffl 2001). Relative quantification was performed by simultaneous quantification of *GAPDH* and *18S* gene expression. The primers used for RT–qPCR analyses are listed in Table 1.

Muscle glutathione (GSH)

Muscle GSH was analyzed by a commercial company (Vitas AS, Oslo, Norway) using a reagent kit originally intended for measurement of total homocysteine in plasma by HPLC (Cat#195-4075, Bio-Rad). The kit was modified and validated for quantification of total glutathione in tissues, with a detection limit (LOQ) of 0.04 mmol/L. All samples were analyzed in triplicate (CV% <2%).

Blood sampling and analysis

Venous blood was drawn in the morning after overnight fasting. Heparin and EDTA coated tubes were centrifuged at 1500 *g* for 10 min at 4°C. Heparin plasma destined for vitamin C analysis was mixed in equal volumes with metaphosphoric acid before freezing; the further analysis procedure is described by Karlsen et al. (2005). Vitamin E was analyzed in EDTA plasma, as described by Bastani

Table 1. Human primer sequences used for RT-qPCR.

Gene name (accession no.)	Sense	Antisense
CRYAB (NM_001885)	GTCAACCTGGATGTGAAGCA	TTTTCCATGCACCTCAATCA
HSPA4 (NM_002154)	TTAAGTCCAAAATCCGTGCAT	CTGAAGCATTTGCACTCATCA
S0D2 (NM_000636)	CCCTGGAACCTCACATCAAC	GGTGACGTTCAGGTTGTTCA
GPx1 (NM_000581)	ACGATGTTGCCTGGAACTTT	TCGATGTCAATGGTCTGGAA

et al. (2012). Plasma glutathione analyses (GSH and GSSG) followed procedures as described by Sakhi et al. (2006). Uric acid and total antioxidant status (TAS) in plasma was measured by a colormetric enzymatic method on an automatic analyzer (MaxMat PL, MaxMat S.A., Montpellier, France). Detection of uric acid was achieved by reagents from MaxMat S.A. (Cat#RM URAC0200V). To ensure adequate quality, multiparametric control sera, two levels of controls, one close to the normal patient values (Maxtrol N, Cat#RM MNCO0030V) and a second representative of pathologic values (Maxtrol P, Cat#RM MPCO0030V) from MaxMat S.A. was used. TAS was measured by incubating the sample with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonatel]). Quantification of TAS was achieved by reagents and standards (Cat#NX 2332) and control (Cat#NX 2331) from Randox Laboratories (Randox Laboratories Ltd., Crumlin, UK).

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 effects of training (pre-post-training intervention and acute exercise session) and supplementation, and a Holm–Sidak multiple comparisons test was applied for post hoc analyses. Unpaired *t*-test was used to evaluate differences between groups in participant characteristics and variables for exercise intensity (Percentage of HF_{max} and VO_{2max}, and lactate) in the acute experiment. Correlations were tested using the Pearson product-moment correlation coefficient test. Effect sizes for mRNA data was calculated and reported using Cohen's *d*. An effect size of 0.2 was considered small, 0.5 medium, 0.8 large, and 1.3 very large. Figures display max–min values, 25th and 75th quartiles and medians (boxplot). Outliers defined by Tukey's rule are shown in figures. The level of significance was set to $P < 0.05$, and *P* values are reported as multiplicity-adjusted *P* values. Trends were defined as $P = 0.05$–0.1. Graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses.

Results

The two groups were not different at baseline (pre-training) regarding training experience, VO_{2max}, age, body weight or height (Table 2). Furthermore, the groups completed the same number of training sessions, exercised with equal training intensity (heart rate monitoring and perceived exertion), and ingested similar levels of energy (data not shown; see ref. Paulsen et al. 2014).

Vitamin C and E concentration in plasma

At baseline, the groups did not differ in plasma vitamin C or E concentration. The vitamin C and E group increased basal plasma vitamin C and E concentration after 11 weeks of supplementation ($P = 0.004$), with higher concentrations compared to the placebo group (43 ± 53 vs. 3 ± 32%, and 31 ± 27 vs. 8 ± 28%, for vitamin C and E, respectively; $P < 0.001$; Fig. 1).

Acute response to supplementation and exercise

The vitamin C and E supplementation increased plasma vitamin C concentration the first hour after ingestion (Fig. 2), and it remained elevated for 3 h after ingestion ($P = 0.047$). The placebo group did not show any significant changes in plasma vitamin C concentration during or after the exercise session.

The exercise intensity during the acute exercise experiment was not different between groups (Table 3): Mean of all intervals; percentage of HR_{max}; 93 ± 1 versus

Table 2. Baseline characteristics of the participants included in the study.

	Group	
Variable	Vitamin C and E	Placebo
Age (years)		
All	24 ± 4	23 ± 3
Males	25 ± 5	24 ± 4
Females	24 ± 4	22 ± 1
Body weight (kg)		
All	71 ± 13	70 ± 13
Males	79 ± 13	80 ± 10
Females	63 ± 8	60 ± 6
Height (cm)		
All	176 ± 11	176 ± 10
Males	184 ± 10	183 ± 5
Females	168 ± 4	168 ± 6
Training hours per week		
All	3.8 ± 1.8	5.4 ± 5.8
Males	3.6 ± 1.5	6.2 ± 6.9
Females	3.9 ± 2.2	4.2 ± 3.8
VO_{2max} (mL/kg/min)		
All	54.6 ± 8.6	54.2 ± 7.4
Males	59.9 ± 8.4	58.0 ± 6.6
Females	50.1 ± 5.7	49.8 ± 6.0

Values are mean ± standard deviations.

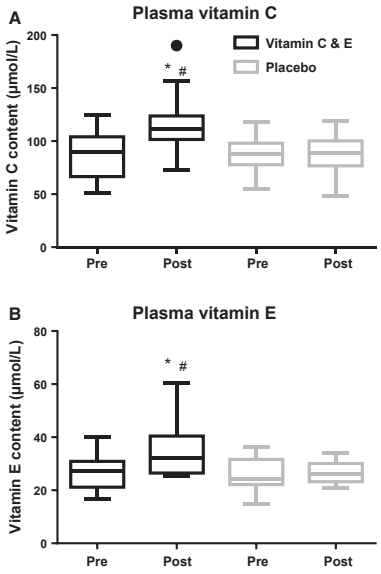

Figure 1. Plasma vitamin C content (ascorbic acid + dehydroascorbic acid) (A) and plasma vitamin E content (B) for the vitamin C and E- (black) and placebo group (gray) before and after 11 weeks of training. ●:Outlier (Tukey's rule); #:difference between groups ($P < 0.05$); *:different from pre ($P < 0.05$).

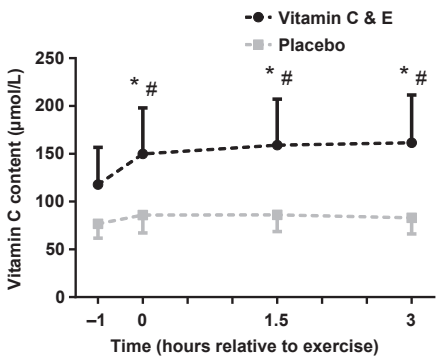

Figure 2. Plasma vitamin C (ascorbic acid + dehydroascorbic acid) content for the vitamin C and E- (black) and placebo group (gray) after ingestion and the acute exercise bout exercise (at time point 0); #:difference between groups ($P < 0.05$); *:different from pre (time point −1; $P < 0.05$).

92 ± 2%, percentage of VO_{2max}; 89 ± 3 versus 88 ± 3%, blood lactate; 4.2 ± 1.1 versus 3.9 ± 0.9 mmol/L for the vitamin C and E and placebo group, respectively.

Ninety minutes after exercise, no changes were observed in skeletal muscle glutathione (GSH) content (Fig. 3).

A tendency ($P = 0.082$) toward decreased cytosolic NFκB p65 content was observed 90 min after exercise, with no effect of supplementation (Fig. 4A). No significant changes were observed 90 min after exercise in nuclear NFκB p65 (Fig. 4B) and cytosolic IκBα content (Fig. 4C).

Ninety minutes after exercise, the vitamin C and E group showed decreased αB-crystallin content in the cytosolic fraction compared to pre values ($P = 0.021$) and placebo ($P = 0.002$; Fig. 5A). αB-crystallin in the cytoskeletal fraction showed low or non-detectable levels independently of group (data not shown). The vitamin C and E group showed decreased HSP27 content in the cytosolic fraction 90 min after the interval bout compared to pre-values ($P = 0.030$; Fig. 5B) and the placebo group (−26 ± 20 vs. 9 ± 29%, respectively; $P = 0.014$). A slight tendency ($P = 0.117$; Fig. 5C) to increased HSP27 amount was observed in the cytoskeletal fraction 90 min after exercise in both groups, with no effects of supplementation.

Table 3. Intensity variables during the acute exercise bout.

Variable	Interval 1	Interval 2	Interval 3	Interval 4
% HF_{max}				
Vitamin C and E	90 ± 1	92 ± 1	94 ± 1	95 ± 1
Placebo	90 ± 2	91 ± 2	92 ± 1	93 ± 1
% VO_{2max}				
Vitamin C and E	86 ± 3	88 ± 3	90 ± 4	92 ± 5
Placebo	87 ± 4	88 ± 4	89 ± 3	89 ± 5
La^- (mmol/L)				
Vitamin C and E	3.3 ± 1.0	3.9 ± 0.8	4.4 ± 1.3	5.2 ± 1.7
Placebo	3.1 ± 0.9	3.9 ± 0.9	4.2 ± 1.2	4.3 ± 1.2

Values are mean ± standard deviations.

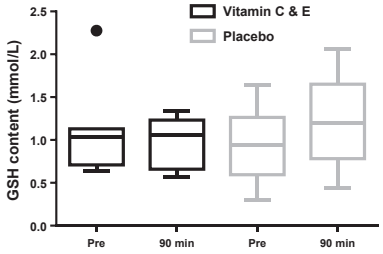

Figure 3. Muscle GSH content for the vitamin C and E- (black) and placebo group (gray) pre and 90 min after exercise. ●:Outlier (Tukey's rule).

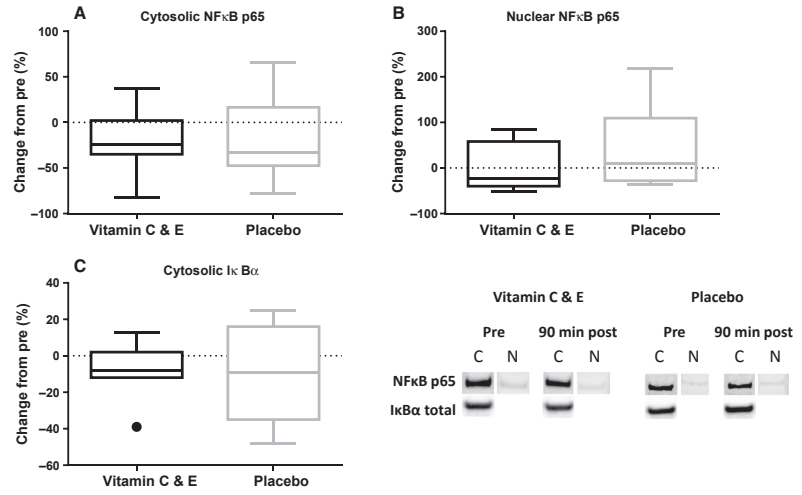

Figure 4. Relative change in muscle cytosolic NFκB p65 (A), nuclear NFκB p65 (B) and total cytosolic IκB (C) measured by protein immunoblot for the vitamin C and E- (black) and placebo group (gray) 90 min after exercise. ●:Outlier (Tukey's rule). C, cytosolic fraction; N, nuclear fraction.

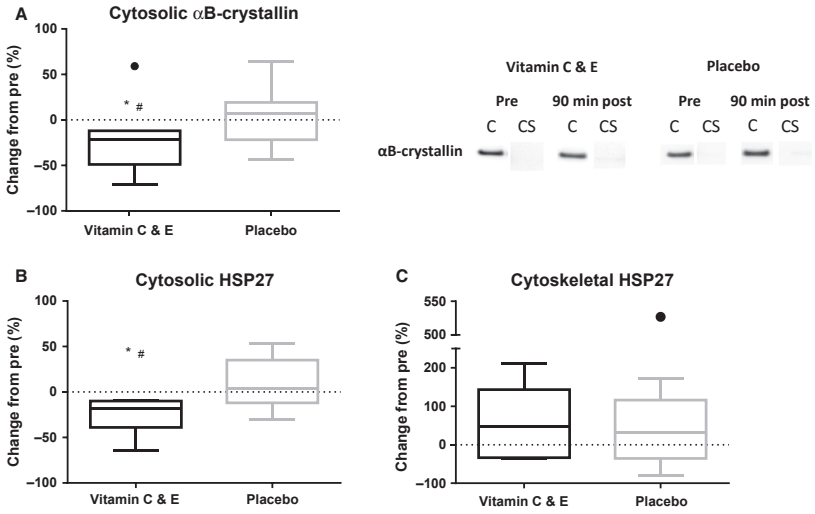

Figure 5. Relative change in muscle cytosolic αB-crystallin (A), measured by protein immunoblot, and cytosolic HSP27 (B) and cytoskeletal HSP27 (C) measured by ELISA for the vitamin C and E- (black) and placebo group (gray) acutely after exercise. ●:Outlier (Tukey's rule); #:difference between groups ($P < 0.05$); *: different from pre ($P < 0.05$). C, cytosolic fraction; CS, cytoskeletal fraction fraction.

Except for a tendency to increased *GPx1* mRNA expression 90 min postexercise ($P = 0.096$; Fig. 6A), *SOD2* (mnSOD), *HSPA*4 (HSP70) or *CRYAB* (αB-crystallin) mRNA expression did not change significantly relative to pre-exercise values (Fig. 6B–D). Although the expression of the investigated genes was not significantly affected by the vitamin C and E supplementation, effect size calculations revealed effect sizes >0.6 between groups; *GPx1*

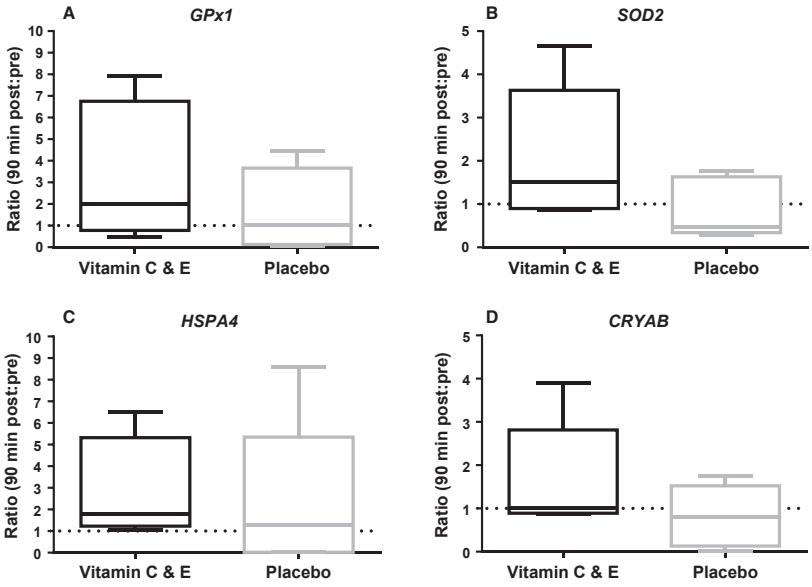

Figure 6. Muscle mRNA expression-ratio (Post:Pre) for *GPx1* (A), *SOD2* (B), *HSPA4* (C) and *CRYAB* (D) measured by RT-qPCR for the vitamin C and E- (black) and placebo group (gray) 90 min after exercise (n = 10).

ES = 0.64 (medium), *SOD2* *ES* = 1.01 (large), *CRYAB* *ES* = 0.82 (large), *HSPA4* *ES* = 0.64 (medium).

Chronic effects of training and supplementation on skeletal muscle variables

After 11 weeks of endurance training, muscle GPx1 levels were decreased by −11 ± 14% in the vitamin C and E group and by −19 ± 18% in the placebo group ($P < 0.001$; Fig. 7A), with no effects of supplementation. No changes were observed for muscle mnSOD levels (Fig. 7B), muscle GSH levels (Fig. 7C), or cytosolic HSP70 levels (Paulsen et al. 2014). The αB-crystallin levels were decreased by −10 ± 14% in the vitamin C and E group and −9 ± 17% in the placebo group ($P < 0.001$; Fig. 8A), with no effects of supplementation. After 5–6 weeks of training (at the commencement of the acute exercise-induced stress test), the vitamin C and E group had unchanged HSP27 levels compared to pre-values (6 ± 17%), while the placebo group displayed a 24 ± 32% reduction ($P = 0.016$). In the end of 11 weeks of training, HSP27 amount was decreased by −17 ± 17% in the vitamin C and E group and −14 ± 33% in the placebo group ($P < 0.002$; Fig. 8B), with no effects of supplementation.

Chronic effects of training and supplementation on plasma variables

A significant decrease in uric acid was observed in the vitamin C and E group by −24 ± 21% compared to the placebo group (6 ± 27%; $P < 0.001$; Table 4) after training. Plasma GSH increased after 11 weeks of training in both groups ($P = 0.003$; Table 4), with no effect of supplementation. Oxidized glutathione (GSSG) did not change after training, whereas total GSH (GSH + GSSG) increased after training in both groups ($P = 0.003$; Table 4), with no effect of supplementation. The ratio between GSH:GSSG did not change after training (Table 4). Total antioxidant capacity (TAS) did not change after training or supplementation (Table 4).

Correlations

The changes observed in mRNA expression acutely after the standardized exercise bout performed in the middle of the training period did not correlate with changes in protein levels during the entire training period for either GPx1, mnSOD, HSP70 or αB-crystallin.

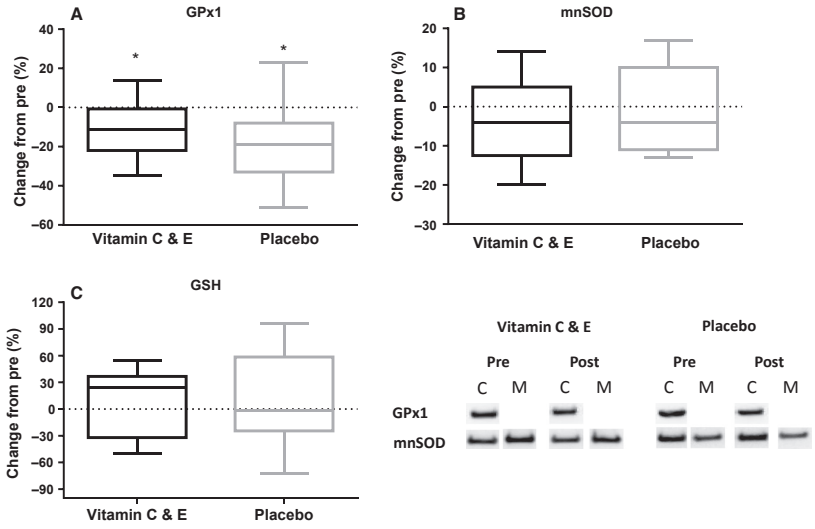

Figure 7. Changes in protein levels for GPx1 (A), mnSOD (B) and GSH (C) measured by protein immunoblot for the vitamin C and E- (black) and placebo group (gray) after 11 weeks of training. *:different from pre ($P < 0.05$). C, cytosolic fraction; M, membrane fraction.

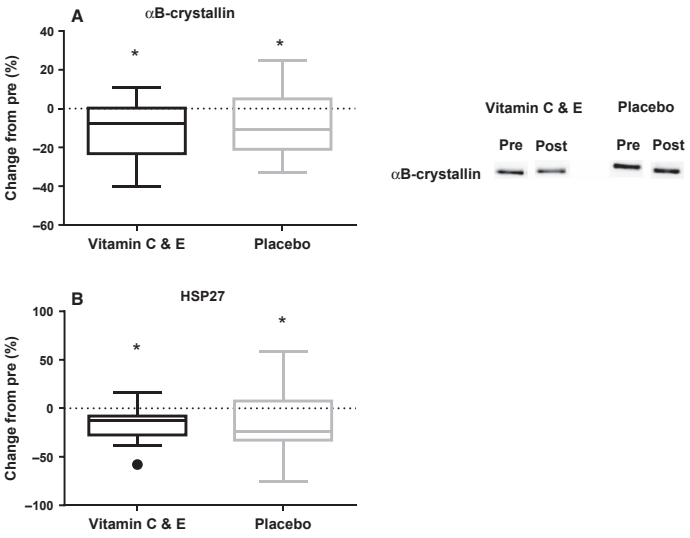

Figure 8. Changes in protein levels for αB-crystallin (A), measured by protein immunoblot, and HSP27 (B) measured by ELISA for the vitamin C and E- (black) and placebo group (gray) after 11 weeks of training. ●:Outlier (Tukey's rule); *:different from pre ($P < 0.05$).

Discussion

The effects of antioxidants on the adaptations to exercise have been studied by a number of research groups, often with conflicting results. By means of a double blind randomized placebo-controlled trial we investigated the effects of vitamin C and E supplementation on the acute stress response to exercise and training adaptations in the

Table 4. Changes in plasma variables before and after 11 weeks of endurance training.

Variable	Pre-training	Post-training	%-Change	Level of significance		
				Group	Training	Interaction
GSH (μmol/L)						
Vitamin C and E	2.4 ± 0.5	2.8 ± 0.7	23 ± 38	$P = 0.991$	$P = 0.003$	$P = 0.577$
Placebo	2.3 ± 0.7	2.9 ± 1.1	32 ± 53			
GSSG (μmol/L)						
Vitamin C and E	0.08 ± 0.05	0.1 ± 0.08	50 ± 101	$P = 0.479$	$P = 0.362$	$P = 0.223$
Placebo	0.08 ± 0.05	0.08 ± 0.04	18 ± 77			
Total GSH (μmol/L)						
Vitamin C and E	2.5 ± 0.6	2.95 ± 0.6	23 ± 37	$P = 0.950$	$P = 0.003$	$P = 0.637$
Placebo	2.4 ± 0.7	3.0 ± 1.1	31 ± 51			
GSH:GSSG						
Vitamin C and E	36.2 ± 18.5	41.4 ± 28	38 ± 109	$P = 0.659$	$P = 0.136$	$P = 0.592$
Placebo	35.8 ± 16	46.8 ± 29.8	59 ± 115			
Uric acid (μmol/L)						
Vitamin C and E	191.5 ± 53.6	146.1 ± 50.6	−24 ± 21	$P = 0.06$	$P = 0.021$	$P < 0.001$
Placebo	200.3 ± 61.7	210.8 ± 77.8	6 ± 27			
TAS (mmol/L)						
Vitamin C and E	1.40 ± 0.10	1.38 ± 0.08	−2 ± 4	$P = 0.101$	$P = 0.830$	$P = 0.467$
Placebo	1.45 ± 0.10	1.46 ± 0.07	1 ± 6			

GSH, reduced glutathione; GSSG, oxidized glutathione; Total GSH, GSH + GSSG; TAS, total antioxidant status.
Values are mean ± standard deviations.

antioxidant systems and heat shock proteins after 11 weeks of endurance training. The main findings were that the acute reductions in cytosolic αB-crystallin and HSP27 content, as well as the stress-related gene expression response, were more pronounced in the vitamin C and E group than in the placebo group 90 min after exercise. Furthermore, we observed that GPx1, αB-crystallin, and HSP27 decreased after 11 weeks of endurance training, but antioxidant supplementation did not have any beneficial or detrimental effects on these variables. Vitamin C and E supplementation did, however, markedly decrease uric acid content in plasma. Finally, endurance training per se increased GSH content in plasma.

Acute response to exercise

The intensity during the interval session was high and similar for both groups, as indicated by the heart rate, VO_2, and blood lactate levels. Despite the high exercise intensity, no detectable degradation of IκBα or translocation of NFκB p65 to the nuclei was observed 90 min after exercise. Consequently, the stress induced during the exercise session appeared insufficient in activating the NFκB pathway. Alternatively, the timing of the biopsy (90 min after exercise) might not have been optimal for detecting NFκB activation, since NFκB p65 in the nucleus has been shown to peak 2 h after exhaustive running in rats (Hollander et al. 2001).

We observed no significant increase in antioxidants (*GPx1* and *SOD2*) or HSP (*CRYAB* and *HSPA4*) mRNA expression after the exercise session. However, there might be a possibility that we missed the alterations of these mRNAs, which could occur at a later time point, and the low number of participants included in these analyses also reduced the statistical power. Interestingly, effect sizes indicate that the vitamin C and E group, in general, had higher mRNA expression of *GPx1*, *SOD2*, *CRYAB*, and *HSPA4*, relative to the placebo group after the interval session. In line with these observations, Yfanti et al. (2012) reported overall higher basal GPx1 mRNA expression in the antioxidant supplemented group.

A larger stress response to the interval session in the vitamin C and E group was further supported by the significant reductions in soluble αB-crystallin and HSP27 observed 90 min after exercise. The decreased αB-crystallin and HSP27 levels in the cytosolic fraction indicate a rapid translocation to stressed cell structures, as seen in experiments with eccentric exercise (Koh and Escobedo 2004; Paulsen et al. 2009).

Seemingly, the increased stress response observed in the vitamin C and E group is in contrast to our initial hypothesis of decreased exercise-induced stress by antioxidant supplementation. However, the interval session was conducted after 5–6 weeks of combined supplementation and training, and we suggest that the placebo group tolerated the stress from the intervals better than the vitamin

C and E group because of more adequate training adaptations in this period. More precisely, mitochondrial adaptations to the endurance training was attenuated in the vitamin C and E group (Paulsen et al. 2014), and this might have caused higher exercise-induced stress in the vitamin C and E group than in the placebo group. Furthermore, decreased muscle HSP27 levels during the first 5–6 weeks of training only observed in the placebo group supports a more rapid and adequate training adaptation in the placebo group compared to the vitamin C and E group (discussed later).

Chronic adaptations to training and supplementation on skeletal muscle variables

Antioxidant systems

No observable increases in mnSOD levels during 11 weeks of training was surprising, as endurance training has been shown to increase mnSOD in animals (Higashida et al. 2011) and trained humans (Morton et al. 2008; Yfanti et al. 2010). Lack of increases in mnSOD might, however, be explained by initially high levels in our well-trained participants. As a consequence of no changes in mnSOD levels with training, it could not be expected to find any blunting effect of vitamin C and E supplementation. A possible interference from supplementation could, therefore, be hidden by initial high protein levels, and it might be a chance that untrained individuals would experience different outcomes than in this study.

Contrary to mnSOD, GPx1 was decreased in both groups after training. These findings are, in agreement with Gliemann et al. (2013), who reported that 8 weeks of high-intensity endurance training decreased GPx1 protein content in initially inactive elderly men, and with no effects of resveratrol supplementation.

As recently published, vitamin C and E supplementation blunted the endurance training induced increase in mitochondrial content (as measured by COX4 protein content) in the same participants as in the present study (Paulsen et al. 2014). The fact that we did not observe any group differences in mnSOD (and GPx1) content in the present study, indicates that oxidative enzymes in the mitochondria and mnSOD are differently regulated. We suggest that the increases in mitochondrial content in the placebo group, with no increases in mnSOD, indicate a better mitochondrial function with less electron leakages during exercise. The improved mitochondrial function might in addition reduce hydrogen peroxide (H_2O_2) in cytosol (Venditti et al. 2014); which might contribute to the observed reduction in GPx1 levels. In the vitamin C and E group, the antioxidant supplementation hampered the mitochondrial adaptations, but in contrast to the placebo group, the antioxidant function of the supplements induced more substrate for RONS scavenging. This can, at least in theory, explain decreased GPx1 content in the vitamin C and E group. Unchanged GSH levels measured in muscle homogenate could be explained by the fact that increases in GSH have been seen in muscles containing mostly type 2 fibers (Sen et al. 1992; Leeuwenburgh et al. 1997). As our participants had 51% type 2 fibers in the investigated muscle (Paulsen et al. 2014), any increases may have been camouflaged by potential fiber-specific changes.

Heat shock proteins

The acute stress response to the high-intensity interval session observed in the vitamin C and E group normally results in increased expression of heat shock proteins (Morton et al. 2009b). This was, however, not reflected in the biopsies taken after the training period as αB-crystallin and HSP27 levels were decreased in both groups. Cytosolic HSP70 protein levels were unchanged after the training period, but two participants (one in each group) displayed a very large increase in HSP70 after training (could be defined as outliers). When removing these values from the data set, a significant decrease ($P < 0.001$) was observed in HSP70 levels as well.

Decreased HSP levels during the training period was unexpected, because previous studies have found that endurance training increases HSP levels in skeletal muscles (Yoshioka et al. 2003; Morton et al. 2008; Morton et al. 2009a). Interestingly, we observed that the vitamin C and E group had unchanged HSP27 levels 5–6 weeks into the training period (data from the acute exercise bout) compared to pre-values, whereas the placebo group had decreased HSP27 levels at this time point. Since the HSPs play an important role in cell stress homeostasis, these findings support our hypothesis that more exercise stress was generated in the vitamin C and E group during the initial 5–6 weeks of training. Nevertheless, because the HSP27 levels were decreased similarly in both groups after 11 weeks of endurance training, it seems like this effect was transient and related to the first weeks of supplementation.

Chronic adaptations of training and supplementation on plasma variables

Due to high plasma concentration and antioxidant function of uric acid (Ames et al. 1981; Glantzounis et al. 2005), plasma total antioxidant status (TAS) measurements are highly dependent on the uric acid concentra-

tion. Although, plasma uric acid levels decreased in the vitamin C and E group after training, TAS did not. This was probably because of the increased plasma vitamin C levels, thus, the overall antioxidant capacity in plasma was held stable.

In contrast to muscle GSH, plasma GSH and total plasma GSH levels (GSH + GSSG) increased significantly as an effect of training. This is in agreement with a previous cross-sectional study reporting higher levels of plasma GSH in trained compared to untrained individuals (Kretzschmar et al. 1991). There is a lack of studies investigating plasma GSH after endurance training, but a few animal studies report no changes in response to training (Leeuwenburgh et al. 1997; Ohkuwa et al. 1997). Despite being trained before entering the study, the participants in the present study increased plasma GSH and total plasma GSH over the training period. This indicates that the training stress was sufficient to induce upregulation of plasma GSH levels even in trained subjects. The vitamin C and E supplementation induced no additional effects on plasma GSH. However, vitamin C supplementation alone has been demonstrated to increased GSH in red blood cells (Johnston et al. 1993) and lymphocytes (Lenton et al. 2003). Thus, we cannot exclude a possible effect on other GSH fractions.

Conclusion

The present study investigated the effects of vitamin C and E supplementation on acute responses to exercise and long-term (11 weeks) adaptations to endurance training on antioxidant systems and heat shock proteins. The results show that vitamin C and E supplementation did not significantly affect the acute activation of the NFκB pathway. However, the acute reductions observed in cytosolic αB-crystallin and HSP27 together with the more pronounced expression of stress genes after the standardized interval session indicate that the exercise-induced stress measured in the middle of the intervention was less tolerated in the vitamin C and E group. The training decreased muscle GPx1, αB-crystallin and HSP27 levels, whereas no changes were observed in mnSOD and muscle GSH levels. Plasma GSH increased in both groups. Vitamin C and E supplementation decreased plasma uric acid levels, but plasma total antioxidant status was stable, likely because of the concomitant increase in plasma vitamin C and E levels. We conclude that vitamin C and E supplementation did not negatively affect the training-induced adaptations in muscle antioxidant systems or heat shock proteins in healthy previously trained participants. However, indications of less tolerability toward exercise-induced stress in the vitamin C and E group could in the long-term, or during periods of very intense training, delay recovery, and not cause optimal gains in physical performance.

Conflict of Interest

None declared.

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Paper 4

Cumming, K. T., T. Raastad, A. Sørstrøm, M. P. Paronetto, N. Mercatelli, D. Caporossi, and G. Paulsen. Vitamin C and E supplementation does not alter heat shock proteins or endogenous antioxidants in skeletal muscles after 12 weeks of strength training.