The Exercise-Induced Expression of Heat Shock Proteins in Human Skeletal Muscle: The Role of Elevated Muscle and Core Temperature and the Influence of Training Status

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Abstract

Skeletal muscle adapts to the stress of contractile activity with a change in gene expression to yield a family of highly conserved cytoprotective proteins known as heat shock proteins (HSPs). These proteins function to restore cellular homeostasis and to protect the cell against further insults. The exercise-induced stress response of rodent muscle is now relatively well defined. Comparable data from human studies, however, are extremely limited and the stress response of human skeletal muscle is far from understood. The main aims of this thesis were to characterise the time-course and magnitude of response of the exercise-induced production of the major HSP families in human skeletal muscle. The role of increased muscle and core temperature in contributing to the exercise-induced production of HSPs was also investigated. Finally, the effects of training status on baseline muscle content of the major HSP families and on the magnitude of the exercise-induced stress response was also examined.

All of the exercise related studies undertaken in this thesis employed a 45 min running exercise protocol on a motorised treadmill at an intensity corresponding to the lactate threshold. This protocol was characterised as 'non-damaging' in nature as it resulted in no overt structural or functional damage to the muscle of young untrained (27 ± 5 years), recreationally active (25 ± 2 years) or aerobically trained male subjects (27 ± 6 years), as evidenced by indirect indicators of muscle damage such as circulating levels of creatine kinase and maximal quadriceps isometric muscle force.

The time-course and magnitude of the exercise-induced response of the major HSP families were characterised in an active young (24 ± 4 years) male population. Muscle biopsies were obtained from the vastus lateralis muscle immediately prior to and at 24 h, 48 h, 72 h and 7 days post-exercise. Exercise induced significant and individually variable increases in HSP70, HSC70 and HSP60 content with peak increases typically occurring at 48 h post-exercise. In contrast, exercise did not induce significant increases in either HSP27, αB-crystallin, manganese superoxide dismutase (MnSOD) protein content or the activity of superoxide dismutase (SOD) and catalase. When examining baseline protein levels, HSC70, HSP27 and αB-crystallin appeared consistently expressed between subjects whereas HSP70 and MnSOD displayed marked individual variation of up to 3 and 1.5 fold, respectively. These data demonstrate a differential effect of aerobic exercise on specific HSPs. Data also demonstrate an individual variation in both basal HSP levels and in the magnitude of the stress response to acute exercise, which may be related to individual differences in training status.

The role of increased muscle and core temperature in contributing to the exercise-induced production of HSPs were subsequently investigated. Active young males (23 ± 3 years) underwent a passive heating protocol of 1 h duration during which the temperature of the core and vastus lateralis muscle were increased to similar levels as that occurring during exercise. One limb was immersed in a tank containing warm water whilst the contra-lateral limb remained outside the tank and was not exposed to heat stress. Muscle biopsies were obtained from the vastus lateralis of both legs immediately prior to and at 48 h and 7 days post-heating. The heating protocol induced significant increases in rectal and muscle temperature of the heated leg whilst muscle temperature of the non-heated limb showed no significant change following

heating. The heating protocol failed to induce significant increases in muscle content of HSP70, HSC70, HSP60, HSP27, αB-crystallin, MnSOD protein content or the activity of SOD and catalase in either the heated or non-heated leg. Data demonstrate that increases in both systemic and local muscle temperature *per se* appear not to be mediating the exercise-induced production of HSPs and suggest that non-heat-stress factors associated with muscle contractile activity are of more importance in mediating this response.

The influences of aerobic training status on the basal levels of HSPs and on the magnitude of the exercise-induced stress response were also investigated. Muscle biopsies were obtained from the vastus lateralis of young trained (28 ± 6 years) and untrained (29 ± 6 years) male subjects immediately prior to and at 48 h and 7 days post-exercise. When comparing muscles at rest, trained subjects had significantly higher levels of αB-crystallin, HSP60 and MnSOD compared with untrained subjects. Trained subjects also had a tendency for higher levels of HSP70, HSC70 and total SOD activity compared with untrained subjects. In contrast to the active population examined earlier, neither the trained nor untrained subjects exhibited a stress response to exercise. The absence of a stress response in trained subjects is likely due to the increase in baseline defences and the customary nature of the exercise protocol. The absence of a stress response in untrained subjects may be due to the failure of the exercise protocol to elicit a proposed critical threshold intensity that is required to induce increases in muscle HSP content.

This thesis has provided novel data for the literature and has significantly advanced our understanding of the exercise-induced stress response of human skeletal muscle. Future research should examine the effects of exercise intensity on muscle HSP production and investigate the role of reactive oxygen species in contributing to the response. The wider implications of the exercise-induced production of HSPs, such as their potential cytoprotective properties against related and non-related stressors, should also be examined.

Key Words: molecular chaperones, oxidative stress, hyperthermia, stress proteins

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Dedication

This thesis is dedicated to the memory of my father. Thank you for teaching me the importance of living life with a genuine appreciation and enthusiasm, a respect for one another and perhaps most importantly, a smile on your face. For the things that *really* matter in life, you have been by far my biggest mentor. I will be forever grateful.

Declaration

I declare that the work presented in this thesis is entirely my own. Some of this work has been published in European and International Journals and presented at National and International conferences.

This thesis has resulted in the following publications and conference communications:

1. Morton, J.P., Atkinson, G., MacLaren, D.P.M., Cable, N.T., Gilbert, G., McArdle, A., Broome, C. and Drust, B. (2005). Reliability of maximal muscle force and voluntary activation as markers of exercise-induced muscle damage. *European Journal of Applied Physiology*, **94**, 541-548.

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Abbreviations

ADP	adenosine triphosphate
AP-1	activator protein 1
AP-2	activator protein 2
ATP	adenosine diphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celsius
Ca^{2+}	calcium
CAT	catalase
CK	creatine kinase
CoQ	coenzyme Q
CuZnSOD	copper zinc superoxide dismutase
d	days
dH_2O	distilled water
DOMS	delayed onset muscle soreness
DTT	dithiothreitol
EcSOD	extracellular superoxide dismutase
EDL	extensor digitorum longus
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol bis-2-aminoethyl ether-N,N',N',N'-tetraacetic acid
eNOS	endothelial nitric oxide synthase
ESR	electron spin resonance
g	grammes
g	centrifugal force
GPx	glutathione peroxidase
GRP75	glucose regulated protein with molecular weight of 75 kDa
GRP78	glucose regulated protein with molecular weight of 78 kDa
GSH	glutathione
EDL	extensor digitorum longus muscle
Fe	iron
h	hours
HO-1	heme-oxygenase
H_2O	water
H_2O_2	hydrogen peroxide
HSE	heat shock element
HSF1	heat shock transcription factor 1
HSF2	heat shock transcription factor 2
HSF3	heat shock transcription factor 3
HSF4	heat shock transcription factor 4
HSPs	heat shock proteins
HSP27	heat shock protein with molecular weight of 27 kDa
HSP60	heat shock protein with molecular weight of 60 kDa
HSP70	heat shock protein with molecular weight of 72 kDa
HSC70	heat shock protein with molecular weight of 73 kDa
HSP90	heat shock protein with molecular weight of 90 kDa
IL-1	interleukin 1
IL-6	interleukin 6

JNK	c-Jun NH_2 terminal kinase
kDa	kilodalton
LT	lactate threshold
mRNA	messenger ribonucleic acid
mA	milliamp
M	molar
ml	millilitre
mm	millimetre
mmol	millimole
MAPK	mitogen activated protein kinase
mins	minutes
MnSOD	manganese superoxide dismutase
MVC	maximal voluntary contraction
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor kappa B
NK	natural killer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet}$	superoxide
$ONOO^-$	peroxynitrite
$^{\bullet}OH$	hydroxyl
Pi	inorganic phosphate
PC	phosphocreatine
RNA	ribonucleic acid
ROS	reactive oxygen species
RPE	ratings of perceived exertion
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SOD	superoxide dismutase
STAT3	signal transducer and activator of transcription 3
TCS	thermal comfort scale
TEA	triethanolamine
TEMED	NNN'N'- tetramethylethylene-diamine
TNF- α	tumour necrosis factor
Tween	polyoxyethylene-sorbitan monolaurate
µg	microgramme
µl	microlitre
µm	micrometre
$\dot{V}O_{2max}$	maximal oxygen uptake
<	less than
>	greater than

Chapter 1

General Introduction

1.1 BACKGROUND

From a physiological perspective, stress can be viewed as any disruption to homeostasis and can exist at the level of the cell, tissue, organ, organ system or whole organism. At the cellular level, one of the most prominent responses to stress is a rapid change in gene expression to yield a family of highly conserved cytoprotective proteins known as heat shock proteins (HSPs) (Morimoto, 1993; Kiang and Tsokos, 1998, Kregel, 2002). Members of the HSP family are primarily classified according to their molecular weight and include the highly stress inducible and cytosolic HSP70 family, mitochondrial HSP60 and members of the small HSP family such as HSP27 and αB-crystallin. An increased production of HSPs during or following stressful episodes functions to restore cellular homeostasis and to provide cytoprotection against subsequent periods of normally damaging stresses (Welch, 1992; Garramone *et al.*, 1994; Lepore *et al.*, 2000; McArdle *et al.*, 1997; F. McArdle *et al.*, 2004; Maglara *et al.*, 2003; Suzuki *et al.*, 2000).

As their name suggests, HSPs were originally found to be induced in cells exposed to sub-lethal heat shock (Ritossa, 1962; Tissieres *et al.*, 1974). Since these initial findings, a large variety of other stressors including ischemia (Marber *et al.*, 1995), protein degradation (Chiang *et al.*, 1989), hypoxia (Guttman *et al.*, 1980), acidosis (Weitzel *et al.*, 1985), oxidative stress (Adrie *et al.*, 2000), increased intracellular calcium (Welch *et al.*, 1983) and energy depletion (Sciandra and Subjeck, 1983) are now also known to increase HSP expression in a variety of tissues and cell lines. Given that several of the above stressors are characteristic of the homeostatic perturbations occurring in contracting skeletal muscle, it is not surprising that both acute (Locke *et al.*, 1990; Salo *et al.*, 1991; Skidmore *et al.*, 1995; Hernando and Manso, 1997; McArdle *et al.*, 2001) and chronic exercise (Gonzalez *et al.*, 2000; Mattson *et al.*, 2000; Naito *et al.*, 2001; Samelan, 2000) consistently induce increases in HSP content in the skeletal muscle of various animal species.

These findings have recently been extended by data from human studies demonstrating that several HSPs are also up-regulated in human skeletal muscle following a variety of exercise protocols (Febbraio *et al.*, 2002b; Khassaf *et al.*, 2001,

2003; Thompson *et al.*, 2001, 2002, 2003). Despite these initial descriptions, comprehensive data from human studies are extremely limited (a 'Pubmed' literature search combining the terms HSPs, exercise and humans lists less that 15 research publications) and the exercise-induced stress response of human skeletal muscle remains poorly characterised and understood. This is likely due, in part, to the significant methodological difficulties facing investigators in relation to the acquisition of sufficient tissue from the serial muscle biopsies that are necessary to accurately determine the HSP response. Interpretation of data from human studies is also often limited to the response of one particular HSP family (most notably HSP70) and is complicated by the variations in timing of tissue sampling between studies, differing subject characteristics (e.g. age, training status, recent activity levels, gender, nutritional status) and perhaps more importantly, the disparate exercise protocols utilised by investigators (e.g. intensity/duration/mode/damaging/non-damaging).

This is particularly the case in those instances where there is a damaging component to the exercise protocol. In such circumstances, the inflammatory response that occurs in the days following exercise may also contribute to increases in muscle HSP levels as invading phagocytic cells contain relatively high levels of HSPs (Khassaf *et al.*, 2003). In order to avoid the complications of changes in cell type on interpretation of data (i.e. phagocytic cell content), it has therefore been suggested that non-damaging exercise protocols provide a more controlled methodological approach for which to study the exercise-induced regulation of muscle HSP expression (Khassaf *et al.*, 2001; 2003; McArdle *et al.*, 2001; Jackson *et al.*, 2004; Vasilaki *et al.*, 2006).

The disparities between exercise protocols make it extremely difficult to evaluate the precise signal (s) responsible for mediating the stress response during exercise. Exercise-associated hyperthermia, oxidative stress, decreased pH, reduced energy availability, hypoxia, cytokine production and structural damage to the muscle proteins are routinely cited as possible factors initiating the exercise-induced expression of HSPs (Fehrenbach and Niess, 1999; Banfi *et al.*, 2004; Lancaster and Febbraio, 2005; Steinacker and Liu, 2002). The extent of these variables, however, can all be affected by the mode, intensity, duration and contractile nature of the exercise protocol. The exercise-induced production of specific HSPs may therefore

be differentially expressed according to certain characteristics of the chosen exercise stimulus. Nevertheless, given the well documented effect of increased cellular temperature on HSP expression (Locke and Noble, 2002), it is tempting to speculate that it is the increase in contracting muscle temperature and/or core temperature *per se* which is the dominant stressor that is responsible for causing an increased production of HSPs following exercise. This hypothesis, however, has yet to be investigated *in vivo* in human skeletal muscle.

Given their cytoprotective properties, the HSP response to periods of exercise training provides an exciting research area with both obvious performance and health implications. Although an increase in baseline muscle HSP content with exercise training has been observed in animal models (Gonzalez *et al.*, 2000; Mattson *et al.*, 2000; Naito *et al.*, 2001; Samelan, 2000), data from human studies are minimal and inconclusive. The few studies that have investigated this area originate from the same laboratory and have employed short-term training programmes as a study intervention (Liu *et al.*, 1999, 2000, 2004). These studies are limited, however, in their failure to disclose the precise time point of biopsy sampling in relation to the last training session. Considering that the stress response is generally regarded as a transient response required by cells to allow them to adapt to a new level of stress (Locke, 1997), it is therefore possible that many of the reported increases in basal HSP levels simply reflect any changes in exercise intensity/duration from the preceding acute exercise bout rather than a response that is associated with a chronic exercise stimulus.

In addition to a possible training-induced adaptation of basal HSP status, it is also important to consider how training affects the magnitude of the stress response following an acute exercise stress. Several data demonstrate that exercise training might allow an individual to mount a greater and/or faster stress response following an acute bout of stress (Gonzalez *et al.* 2000; Fehrenbach *et al.*, 2000; Campisi *et al.*, 2003). Such data suggest that trained subjects may have an increased ability to recover and regain cellular homeostasis following an acute exercise stress via the increased ability to synthesise HSPs (Gonzalez *et al.*, 2000; Fehrenbach *et al.*, 2000; Campisi *et al.*, 2003). Alternatively, it is possible that the HSP response to acute exercise in the trained state is associated with a reduced stress response as the muscle

is already pre-conditioned with appropriate endogenous defence systems such as increased basal content of HSPs and higher activity of antioxidant enzymes (Smolka *et al.*, 2000; Nething *et al.*, 2004). Precisely how training status influences the basal levels of HSPs and also the magnitude of the stress response of human muscle following an acute exercise stress, therefore remains to be determined.

1.2 Aims, Objectives and Structure of Thesis

The main aims of this thesis are to characterise the time-course and magnitude of response of the major HSP families in human skeletal muscle following an acute bout of non-damaging running exercise. The role of increases in muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle will also be investigated. Finally, the effects of training status on baseline content of the major HSP families in human skeletal muscle and on the magnitude of the exercise-induced production of HSPs following a non-damaging running exercise protocol will also be evaluated.

The above aims will be achieved through the following objectives:

1. To develop a non-damaging running exercise protocol to utilise as an exercise stimulus to initiate the expression of HSPs in human skeletal muscle, where non-damaging is defined as exercise that induces no overt structural or functional damage to the muscle. This will be achieved through the completion of Study 1 and 2 (Chapter 4).

2. To characterise the time-course and magnitude of response of the major HSP families in human skeletal muscle following an acute bout of non-damaging running exercise. This will be achieved through the completion of Study 3 (Chapter 5).

3. To evaluate the role of increases in muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle. This will be achieved through the completion of Study 4 (Chapter 6).

4. To determine the influence of training status on the baseline content of the major families of HSPs in human skeletal muscle. This will be achieved through the completion of Study 5 (Chapter 7).

5. To determine the influence of training status on the magnitude of the exercise-induced stress response of human skeletal muscle following an acute bout of non-damaging running exercise. This will be achieved through the completion of Study 5 (Chapter 7).

Chapter 2

Literature Review

2.1. INTRODUCTION

A major question in the biological sciences is the precise mechanism (s) by which cells respond to a disruption in homeostasis (Morimoto, 1993). Exposure to stress interferes with efficient operations of the cell with negative consequences on the biochemical properties of proteins (Morimoto, 1998). In stressed environments, proteins can unfold, misfold and aggregate which, if left unchecked, will ultimately lead to cell death (Morimoto, 1998). Fortunately, cells have developed a tightly controlled and evolutionary protective response to stress that involves a rapid change in gene expression to yield a class of highly conserved proteins known as heat shock proteins (Kilgore *et al.*, 1998).

The heat shock response, as characterised by the rapid induction of HSPs, was first described by Ritossa (1962). Ritossa initially observed the induction of a set of chromosomal puffs on the polytene chromosomes in the salivary glands of *Drosophila busckii* after exposure to elevated temperatures. Over a decade later, Tissieres and colleagues (1974) subsequently demonstrated that these stress-induced 'puffs' were accompanied by an increased expression of proteins with molecular masses of 26 and 70 kDa. These proteins were originally named as heat shock proteins as they were induced in cells recovering from a transient sub-lethal heat shock during which body temperature was increased approximately 5°C above resting core temperature.

The cellular content of HSPs has also been shown to increase following a large variety of other stressors including ischemia (Marber *et al.*, 1995), protein degradation (Chiang *et al.*, 1989), hypoxia (Guttman *et al.*, 1980), acidosis (Weitzel *et al.*, 1985), oxidative stress (Adrie *et al.*, 2000), increased intracellular calcium (Welch *et al.*, 1983) and energy depletion (Sciandra and Subjeck, 1983). Accordingly, the terms 'stress proteins' and 'cellular stress response' have since been introduced (and are often used interchangeably within the literature for 'heat shock proteins' and 'heat shock response' respectively) so as to reflect the universal nature of the response and the array of stressors known to initiate the expression of these proteins (Locke, 1997).

During the last two decades, the stress of exercise has also been shown to induce increases in HSP expression in cells and tissues of various animal species (Locke *et al.*, 1990; Hernando and Manso, 1997; McArdle *et al.*, 2001; Salo *et al.*, 1991; Skidmore *et al.*, 1995; Walters *et al.*, 1998). These findings have recently been extended by data from human studies (which are the focus of this review) demonstrating that several HSPs are also up-regulated in the skeletal muscle of humans following various types of exercise protocols. These data are still in their infancy, however, and as yet the exercise-induced stress response of human skeletal muscle is far from understood.

The ability of exercise to initiate the heat shock response provides an extremely exciting and important research area for the exercise scientist given that cells demonstrating elevated levels of HSPs following a non-damaging episode of stress are subsequently capable of withstanding normally lethal or damaging stresses (Garramone *et al.*, 1994; Lepore *et al.*, 2000; McArdle *et al.*, 1997; F. McArdle *et al.*, 2004; Maglara *et al.*, 2003; Suzuki *et al.*, 2000). The study of HSPs, in relation to exercise, may therefore increase our understanding of the cellular and molecular mechanisms underpinning the increased protection to contraction-induced damage associated with regular exercise. The possibility of using exercise (as a non-pharmacological approach) as a means to harness a cell's endogenous protective systems to provide 'cross-tolerance' to non-related stressors is also an exciting area that may have important health implications (Locke, 2002). Such research may therefore increase our understanding of the mechanisms by which exercise can provide protection to cells and tissues from 'protein-misfolding diseases' such as cancer, diabetes and various neurodegenerative disorders.

The aim of the present review is to summarise the literature regarding the effect of acute and chronic exercise on HSP expression, where particular reference is given to human skeletal muscle. Where appropriate, relevant data from animal studies will also be presented. The reader is firstly introduced to the generic function of HSPs, the regulation of heat shock gene transcription and the major HSP families found in skeletal muscle. The effects of acute and chronic exercise on HSP expression are then discussed and the possible physiological signals initiating the exercise-induced expression of HSPs are also presented. The review closes by highlighting the possible

biological significance of exercise-induced HSP expression. It is hoped that the need for further research, particularly in relation to the studies undertaken in the present thesis, will be evident throughout.

2.2 General Function of HSPs and Regulation of HSP Expression

2.2.1 Function of HSPs in the unstressed cell

The terms heat shock or stress proteins are somewhat misleading given that several HSPs are constitutively expressed in skeletal muscle where they play an integral role in normal cellular processes. In such circumstances, HSPs function as molecular chaperones necessary for facilitating the correct folding of newly synthesised proteins, preventing the aggregation of aberrantly folded proteins, facilitating the refolding of denatured proteins and for safely transporting proteins to their correct cellular compartment (Welch, 1992; Lancaster and Febbraio, 2005). A comprehensive review of chaperone mediated folding is beyond the scope of the present review and the reader is referred to several authoritative texts (Walter and Buchner, 2002; Wegrzyn and Deuerling, 2005; Mayer and Bukau, 2005). In brief, chaperones bind to hydrophobic residues of misfolded or unfolded proteins thereby preventing their aggregation. Upon ATP hydrolysis, a conformation change in the chaperone triggers the dissociation with the polypeptide providing it with new opportunities for productive folding. As a chaperone, HSPs are therefore considered to perform important 'housekeeping' functions within the cell and are critical for efficient protein turnover (Fehrenbach and Niess, 1999; Fehrenbach and Northoff, 2001). A schematic illustration of the function of HSPs in the unstressed cell is displayed in Figure 2.1A.

2.2.2 Regulation of HSP expression

Heat shock proteins are highly stress-inducible and an increased HSP expression is often diagnostic that the cell has experienced some trauma (Welch, 1992). An

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Figure 2.1 – (A) Function of HSPs in the unstressed cell. Constitutively expressed HSPs bind to and stabilize nascent polypeptides emerging from the ribosome and ensure that they are folded and function correctly. HSPs also transport proteins to their correct site of action (e.g. mitochondria) where other specialised HSPs ensure appropriate folding. **(B)** Function of HSPs in the stressed cell. Following episodes of non-damaging stress, an increased expression of HSPs occurs (see section 2.2.2 for additional text) which can provide protection to subsequent periods of normally lethal stresses (McArdle *et al.*, 2002).

increased production of HSPs is primarily regulated at the level of transcription and the mechanisms by which stress induces an increased expression of HSPs are now relatively well understood (Broome *et al.*, 2003). Expression of HSP genes is dependent on the presence of short nucleotide sequences known as heat shock elements (HSEs) which are generally located upstream in the promoter region of heat shock responsive genes. The HSE is a highly conserved element that contains multiple copies of a 5'-nGAAn-3' core sequence (Liu and Steinacker, 2001). It is the presence of one or more functional HSEs that is the identifying feature of an HSP gene (Locke, 1997). The HSE is the binding site for a constitutively expressed protein known as the heat shock factor (HSF), the transcription factor required to mediate transcription of HSP genes. At present, three different HSFs have been identified in mammalian cells; HSF1 (Rabindran *et al.*, 1991), HSF2 (Schuetz *et al.*, 1991) and HSF4 (Nakai *et al.*, 1997). A fourth HSF, HSF3, has also been identified that is specific to avian species (Nakai and Morimoto, 1993).

HSF1 is the major stress responsive transcription factor in mammalian cells and is the most extensively studied HSF homologue thus far (Santoro, 2000). HSF1 is a 75kDa protein that is constitutively expressed in the cytoplasm and nucleus of unstressed cells (Snoeckx *et al.*, 2001). Under such circumstances, HSF1 exists in an inactive monomer state that co-precipitates with HSP70 and HSP90 (Zuo *et al.*, 1998). During episodes of stress, the freely available HSPs that are present in unstressed situations (see Figure 2.1 B, upper left panel) are subsequently depleted by interactions with unfolded proteins. Those HSPs that are bound with HSF1 will also have a higher affinity for binding for unfolded proteins than HSF1. The accumulation of damaged and/or malfolded proteins therefore displace HSP70 and HSP90 from the HSF1 complex thereby freeing HSF1 for activation (Baler *et al.*, 1992, 1996; Morimoto, 1993; Zuo *et al.*, 1998; McArdle and Jackson, 2002). This sequestration of HSP70 and HSP90 releases HSF1 from its inactive monomeric state, allowing translocation to the nucleus, trimerization, hyperphosphorylation and binding to the HSE of the HSP genes (see Figure 2.2B, upper right panel). Recent studies have also demonstrated that HSF1 is able to directly sense stress (e.g. heat and oxidative stress), form homotrimers and bind to the HSE (Ahn and Thiele, 2003).

The process of trimerization and DNA binding is termed 'HSF activation' (Fehrenbach and Niess, 1999) which can occur within minutes of heat shock (Locke *et al.*, 1995a), hypoxia (Beckmann *et al.*, 1990), ATP depletion (Benjamin *et al.*, 1992), changes in pH (Petronini *et al.*, 1995), metabolic inhibitors (Benjamin *et al.*, 1992), oxidative stress (McDuffee *et al.*, 1997) and exercise (Locke *et al.*, 1995a). In a negative feedback loop mechanism, the increased expression of HSPs will eventually accumulate to a level at which 'free' HSPs can reform the complex with HSF1 thus reverting the latter back to its inactive monomeric form (Locke, 1997; Ho and Westwood, 2002).

2.2.3 Function of HSPs in the stressed cell

The increased expression of HSPs following stressful episodes functions to restore cellular homeostasis and to provide cytoprotection against further insults (Welch, 1992). An increased cellular content of HSPs is thought to promote cellular recovery by binding with misfolded and unfolded proteins and facilitating the refolding of these proteins when cellular conditions become more favourable (Broome, 2003). In this case, the stress-induced expression of HSPs act in a manner analogous to their chaperone function and reflects the operation of a feedback system that responds to increases in misfolded proteins by elevating the synthesis of the chaperones that help them refold (Dobson, 2003).

Direct evidence for protective effects of increased HSP content is provided in those studies employing transgenic approaches whereby cells and tissues that over-express HSPs show considerable protection to normally damaging stresses (Marber *et al.*, 1995; A.McArdle *et al.*, 2004). An increased content of HSPs following mild or non-damaging stresses (i.e. pre-conditioning) can also provide cytoprotection against subsequent periods of normally lethal or damaging stresses (Garramone *et al.*, 1994; Lepore *et al.*, 2000; McArdle *et al.*, 1997; Maglara *et al.*, 2003; F.McArdle *et al.*, 2004; Suzuki *et al.*, 2000). A schematic illustration of the function of HSPs in the stressed cell is displayed in Figure 2.1B. Many HSPs also have specialised functions in both the stressed and unstressed cell. This is discussed in the following section.

2.3 HSP FAMILIES

Heat shock proteins can be classified into a number of HSP 'families' based on their molecular mass. Several HSPs are expressed in skeletal muscle, the most prominent of which include the small HSPs (ranging from 8-32 kDa in size), HSP60 (60 kDa), HSP70 (70 kDa) and HSP90 (90 kDa). An overview of the location and specific functions (where known) of these proteins follows. A summary of the classification of HSPs according to size, location and function is also provided in Table 2.1.

2.3.1 Ubiquitin

Ubiquitin is the smallest HSP (molecular weight of 8 kDa) that is reportedly expressed in human skeletal muscle (Thompson and Scordillis, 1994). It is a highly conserved protein that is constitutively expressed in the cytosol of the unstressed cell. Ubiquitin is considered an HSP as it contains a heat shock element in its promoter region (Bond and Schlesinger, 1986) and is, thus, stress inducible in response to heat shock and other stresses such as cellular injury (Powers *et al.*, 2001) and damaging forms of exercise (Thompson and Scordillis, 1994).

Ubiquitin's primary role is in both chromatin structure and in protein degradation events (Welch, 1992). Many intracellular proteins that are to be degraded are first covalently modified by the addition of ubiquitin. Once ubiquitin conjugated, these proteins are then targeted for degradation via the ubiquitin-proteasome pathway, the major system in muscle for identifying and degrading damaged/denatured proteins (Glickman and Ciechanover, 2001). In this system, proteins that cannot be rescued are covalently attached to polyubiquitin chains and are thereby targeted for degradation by the 26S proteasome in the cytosol (Glickman and Ciechanover, 2001). Proteins that are to be degraded are presented to ubiquitin by the molecular chaperones HSP40/70 only after repeated attempts at refolding have failed (Goldberg, 2003). The observed increases in ubiquitin levels following stress are therefore to facilitate the targeting and removal of damaged and denatured proteins. The ubiquitin-proteasome pathway is therefore considered as an essential mediator of muscle remodelling during both acute and chronic exercise (Reid, 2005).

2.3.2 HSP27

HSP27 (or HSP25 in rodents) is localized in the cytosol in the unstressed cell and is translocated to within or around the nucleus following stress (Arrigo and Welch, 1987). HSP27 is less universally conserved than other HSPs and accumulates with slower kinetics and is synthesised for a longer time after stress (Landry *et al.*, 1991). Although the precise function (s) of HSP27 remain unclear, it has been shown to be involved in microfilament stabilization (Lavoie *et al.*, 1993), signal transduction (Gabai and Sherman, 2002), growth (Welch, 1992), differentiation and transformation processes (Arrigo and Landry, 1994) and in providing protection against thermal (Locke, 1997) and oxidative stress (Escobedo *et al.*, 2004).

HSP27 has been proposed to play a direct role in protecting skeletal muscle from contraction-induced damage specifically via interactions with cytoskeletal elements (e.g. actin filaments) and in regulation of the glutathione system (Koh, 2002). Studies in humans have demonstrated that HSP27 is increased approximately 2-fold 48 h following damaging contractions of the elbow flexors (Thompson *et al.*, 2001, 2002, 2003). Lengthening contractions of the EDL muscles of mice resulted in reduced levels of z-disk and membrane scaffolding proteins (Koh and Escobedo, 2004), which was subsequently accompanied by phosphorylation of HSP25 and translocation to sites at the z-disk and membrane. Given that HSP25/27 has been shown to protect the cytoskeleton of different cell types against a variety of stresses (Welsh and Gaestel, 1998), it is likely that the observed translocation of HSP25 following contraction-induced damage was an attempt to limit cytoskeletal disruption and to aid in repair of injured structures.

2.3.3 αB-crystallin

αB-crystallin is a 22kDa protein which belongs to a family of crystallins found in vertebrate lenses. It is also expressed in tissues that possess a high mitochondrial content such as cardiac and type I and IIa muscle fibres where it tends to co-localise with HSP27 at the I-band and M-line (Neufer *et al.*, 1998). αB-crystallin functions

as a molecular chaperone to either prevent aggregation of denatured proteins or to facilitate refolding upon the removal of stress (Jakob *et al.*, 1993).

Similar to HSP27, αB-crystallin is also involved in stabilization of actin filaments following stressful insults to the cytoskeleton (Mounier and Arrigo, 2002). It has also been proposed to be involved in regulation of desmin intermediate filaments where it may help stabilise the Z-line (Atomi *et al.*, 1991). The observation of increased expression of both desmin and αB-crystallin in the vastus lateralis of humans at 14 days following a downhill running protocol suggests that these proteins may be involved in a remodelling of the Z-disk structures so as to increase resistance to mechanical stresses (Feasson *et al.*, 2002). It has also recently been demonstrated that αB-crystallin maintains skeletal muscle myosin ATPase activity and prevents its aggregation under heat shock stress (Melkani *et al.*, 2006). Furthermore, transgenic mice overexpressing αB-crystallin are significantly protected against damage at 3 h following lengthening contractions, possibly via maintenance of the intermediate filament network (McArdle *et al.*, personal communication). Together with HSP27, αB-crystallin appears to play an important physiological role which functions to protect the cytoskeleton and contractile machinery during stressful insults, such as exercise, in an attempt to maintain muscle performance.

2.3.4 Heme oxygenase 1 (HO-1)

HO-1 (or HSP32) is the inducible isoform of heme oxygenase and is localised in the cytoplasm of cells. Like ubiquitin, HO-1 is considered an HSP as it contains a heat shock element in its promoter region (Okinaga and Shibahara, 1993). It is, however, most often up-regulated in response to oxidative stress that may not operate through traditional heat shock signalling pathways but may involve nuclear factor kappa B (NFkB) and activator-protein 2 (AP-2) as transcription factors (Tacchini *et al.*, 1995). A major function of HO-1 is to catabolize iron-bound heme into biliverdin and ultimately bilirubin and carbon monoxide (Morse and Choi, 2002). In this way, heme (which is a pro-oxidant) is thus converted to antioxidant metabolites. Evidence for a possible role of HO-1 as a defence against exercise-induced oxidative stress in rats

(Essig and Nosek, 1997; Essig *et al.*, 1997; Hildebrandt *et al.*, 2003), mice (F.McArdle *et al.*, 2004) and humans (Pilegaard *et al.*, 2000) has previously been demonstrated. The protective effect of HO-1 is likely a function of the aforementioned elimination of heme with coincident production of antioxidants (Noble, 2002) and production of specific cellular messengers (Cary and Marletta, 2001) rather than involving chaperone functions.

2.3.5 HSP60

HSP60 is located primarily within the muscle mitochondrial matrix where it is constitutively expressed under normal conditions. It is synthesised in precursor form in the cytoplasm and is then translocated to the mitochondria where it is processed into its mature form enabling it to function (Mizzen *et al.*, 1989). HSP60 plays an important role in normal cell function by facilitating the correct folding and assembly of proteins as they enter the mitochondria and facilitating protein transport across intracellular membranes (Hood *et al.*, 2000, 2002, 2003).

Following episodes of stress, HSP60 is believed to be involved in the stabilization of pre-existing proteins (Broome *et al.*, 2003) and is thought to be able to renature proteins that may have denatured within the mitochondria (Martinus *et al.*, 1995). HSP60 is also stress inducible and is increased in both the soleus muscles of mice (McArdle *et al.*, 2001) and vastus lateralis of humans (Khassaf *et al.*, 2001) following non-damaging exercise protocols. HSP60 has also been shown to be up-regulated in the skeletal muscle of rodents following low frequency chronic electrical stimulation (Ornatsky *et al.*, 1995) and treadmill endurance training (Mattson *et al.*, 2000; Samelan, 2000) presumably to facilitate increased mitochondrial protein import and assist in exercise-induced mitochondrial biogenesis

2.3.6 HSP70

The most highly conserved of HSPs and the most widely studied to date are members of the HSP70 family. The 70 kDa HSPs are very abundant proteins that can account

for as much as 1-2% of total cellular protein under resting conditions (Herendeen *et al.*, 1979). Four major isoforms of the HSP70 family have been identified in mammalian cells (Welch *et al.*, 1989), the most prominent of which include a cognate isoform (referred to as HSC70 or HSP73) and an inducible isoform (referred to as HSP70 or HSP72). These two proteins exhibit extremely high sequence homology (~95%) and share similar biochemical properties (Welch, 1992). These proteins perform a vast array of functions within the cell including interaction with cell signalling pathways (Gabai and Sherman, 2002), mRNA stabilization and degradation (Laroia *et al.*, 1999), assisting in protein degradation (Chiang *et al.*, 1989; Goldberg, 2003), and as regulators of cell death (Samali and Orrenius, 1998; Takayama *et al.*, 2003). Members of the HSP70 family have also been reported to self-regulate their stress induced synthesis through interaction with heat shock transcription factor 1 (see section 2.2). By far the most important role of the HSP70 family, however, is in its function as a molecular chaperone and cytoprotection (Kiang and Tsokos, 1998).

HSP70 is present at low levels in the cytoplasm of the unstressed cell and is considered to be primarily stress inducible (Milarski and Morimoto, 1989). A large body of evidence now exists to suggest HSP70 is the key stress protein involved in cytoprotection (for reviews see Locke and Noble, 2002). The cytoprotective mechanisms of HSP70 are suggested to be similar to its chaperone role where it can maintain correct protein folding and translocation, refold misfolded proteins, prevent protein aggregation and assist in the degradation of unstable proteins (Kregel, 2002). Elevated levels of HSP70 are heavily associated with acquired thermotolerance (Kregel, 2002; Katschinski, 2004) and in providing cross-tolerance to non-related stressors. For example, a prior heat stress in rats (which resulted in increased muscle content of HSP70) provided significant protection against necrosis induced by ischemia and reperfusion (Garramone *et al.*, 1993; Lepore *et al.*, 2000).

Direct evidence for a protective role of HSP70 against a variety of stresses is provided from transgenic models. Marber *et al.* (1995) observed that isolated hearts from transgenic mice overexpressing HSP70 demonstrated increased resistance to ischemic injury. A.McArdle *et al.* (2004) also demonstrated that the recovery of maximal tetanic force of the EDL muscles of mice following a damaging contraction protocol was enhanced in both adult and old mice that were overexpressing HSP70. Elevated

levels of HSP70 in cells or tissues of an exercising organism may therefore permit increased tolerance to the biochemical and physiological stresses that accompany exercise (Locke, 1997).

Although primarily stress inducible, HSP70 is also constitutively expressed in skeletal muscle in a fibre type specific pattern (Locke *et al.*, 1991; Locke and Tanquay, 1996; Hernando and Manso, 1997). Muscles such as the soleus (composed primarily of type I fibres) contain higher amounts of HSP72 than those muscles comprised mainly of type II fibres (e.g. the gastronemius). In muscles composed of mixed fibre types, HSP72 content appears proportional to the percentage of type I fibres (Locke *et al.*, 1991; Locke and Tanquay, 1996). O'Neill *et al.* (2006) recently demonstrated that slower fibre phenotypes are critical for constitutive expression of HSP70. A higher content of HSP70 in these situations is to perhaps facilitate the increased rate of protein turnover that is characteristic of these fibres (Obinata *et al.*, 1981).

HSC70 is constitutively expressed in the cytoplasm of unstressed cells at higher levels than HSP70 and is only slightly increased after episodes of stress such as heat shock (Welch, 1992) and exercise (McArdle *et al.*, 2001). During stress, HSC70 migrates to the nucleus and nucleolus where it may bind with denaturing or unfolding pre-ribosomes possibly facilitating renaturation (Welch and Suhan, 1986). The absence of HSC70 slows down ribosome translocation thereby slowing the rate of protein synthesis (Ku *et al.*, 1995).

The remaining two isoforms of the HSP70 family are the glucose-regulated proteins (which are not heat inducible), referred to as GRP75 and GRP78. GRP78 and GRP75 are located in the sarcoplasmic/endoplasmic reticulum and mitochondria respectively (Pelham, 1986). Mattson *et al.* (2000) observed an increased expression of GRP75 (105% of control levels) in rodent plantaris muscle following 8 weeks of treadmill endurance training, suggesting a possible role in facilitating increased mitochondrial protein import and folding during exercise-induced mitochondrial biogenesis.

2.3.7 HSP90

HSP90 (like HSP70) is also a highly conserved family of proteins which represents 1-2% of total cellular protein under resting conditions (Katschinski *et al.,* 2004). The HSP90 family consists of three proteins: the glucose regulated protein GRP94 (located in the endoplasmic reticulum) and the two closely related cytoplasmic isoforms of HSP90α and HSP90β. HSP90 acts in a chaperone like manner that is involved in the folding and activation of an unknown number of substrate proteins including protein kinases, transcription factors (e.g. HSF1, see section 2.2) and, as most well documented, steroid hormone receptors (Richter and Buchner, 2001). These receptors include estrogen, progesterone, glucocorticoid, testosterone and androgen receptors (Welch, 1992). In the absence of the hormone, HSP90 is thought to bind to the receptor and maintain it in an inactive form thereby preventing its inappropriate interaction with DNA (Dalman *et al.,* 1991). Upon presentation of the hormone, the receptor-HSP90 complex disassociates and the receptor is rendered capable of binding with DNA (Locke, 1997). The formation of these proteins with HSP90 is a prerequisite for their stability and function. Members of the HSP90 family are therefore considered as key players in cellular processes (Katschinski, 2004).

Although HSP90 is one of the most abundant proteins in unstressed mammalian cells, it is also increasingly expressed in response to stress (Welch, 1992). The stress-induced expression of HSP90 is an attempt to prevent the unwanted aggregation of partially unfolded proteins by maintaining them in a folding competent state for refolding (Buchner, 1996; Freeman and Morimoto, 1996). Similarly to HSP70, HSP90 may self-regulate its stress-induced synthesis through its interaction with heat shock transcription factor 1 (Zou *et al.,* 1998). Inhibition of HSP90 function has also been shown to delay and impair recovery from heat shock suggesting that a multi-component chaperone complex involving several HSPs is necessary for optimal protection (Duncan, 2005).

Table 2.1 – Classification of HSPs according to size, location and function. Primary references are also provided indicating whether each HSP is induced in human skeletal muscle by acute exercise. If unknown, primary reference is provided from rodent muscle.

HSP Family	Cellular location	Function (s)	Exercise-Induced	Species/Tissue	Reference
Ubiquitin	Cytosol	Protein degradation/chromatin structure	Yes	Human biceps	Thompson and Scordilis (1994)
HSP27	Cytosol/nucleus	Signal transduction/microfilament stabilization/apoptosis/differentiation/ thermotolerance/cytoprotection	Yes	Human biceps brachii	Thompson *et al.* (2001)
HSP32 (HO-1)	Cytosol	Antioxidant/protection against contraction-induced oxidative stress	Yes	Human vastus lateralis	Pilegaard *et al.* (2000)
HSP60	Mitochondria	Molecular chaperone (chaperonin)/ guides mitochondrial protein import and stabilizes mitochondrial proteins during stress	Yes	Human vastus lateralis	Khassaf *et al.* (2001)
HSP70 (HSP72)	Cytosol/nucleus	Molecular chaperone/thermotolerance/ cytoprotection/renatures proteins following stress/apoptosis	Yes	Human vastus lateralis	Khassaf *et al.* (2001)
HSC70 (HSP73)	Cytosol/nucleus	Constitutively expressed molecular chaperone	Yes	Human vastus lateralis	Jackson *et al.* (2004)
GRP75 GEP78	Mitochondria Sarcoplasmic/Endoplasmic reticulum	Glucose regulated proteins	?	?	?
HSP90	Cytosol	Molecular chaperone involved in folding and activation of protein kinases, transcription factors and steroid hormone receptors	Yes	Rat soleus	Locke *et al.* (1990)

2.4 EXERCISE-INDUCED EXPRESSION OF HSPS

Exercise is now accepted as a valid method by which to induce HSP expression in a variety of animal tissues (Tolson and Roberts, 2005). During the 1990's, a series of studies employing treadmill running rodents provided conclusive evidence for acute exercise as a sufficient stimulus to up-regulate HSP expression in tissues such as the heart (Salo *et al.*, 1991; Skidmore *et al.*, 1995), brain (Walters *et al.*, 1998), liver (Salo *et al.*, 1991) and skeletal muscle (Locke *et al.*, 1990; Salo *et al.*, 1991; Skidmore *et al.*, 1995; Hernando and Manso, 1997).

Data from skeletal muscle now demonstrate that acute periods of varying forms of contractile activity can up-regulate αB-crysatllin (Neufer *et al.*, 1998), HSP25 (Koh and Escobedo, 2004), HO-1 (F.McArdle *et al.*, 2004), HSP60 (McArdle *et al.*, 2001), HSC70 (McArdle *et al.*, 2001), HSP70 (Salo *et al.*, 1991; Hernando and Manso, 1997; McArdle *et al.*, 2001; Milne and Noble, 2002; Kim *et al.*, 2004) and HSP90 (Locke *et al.*, 1990) in various animal species. The exercise-induced stress response of rodent skeletal muscle is now relatively well characterised. Time-course approaches have been adopted following both treadmill running (Hernando and Manso, 1997) and electrical stimulation protocols (McArdle *et al.*, 2001) and have typically demonstrated maximal increases in HSP content within 4-12 hours following exercise. A comprehensive review of the stress response of animal tissue is beyond the scope of the present review and thus the reader is referred to several excellent reviews (Locke and Noble, 2002; McArdle and Jackson, 2002, Liu and Steinacker, 2001; Liu *et al.*, 2006).

Data from human studies also demonstrate that both endurance and resistance exercise can up-regulate HSP expression in human skeletal muscle. Comparison amongst studies, however, is extremely difficult due to the disparate exercise protocols employed by investigators (e.g. mode, duration, intensity, damaging, non-damaging), differing timing of biopsy sampling across studies, the specific muscle examined (e.g. vastus lateralis vs biceps brachii) and differing subject characteristics both within and between studies (e.g. gender, training status, recent activity levels etc). The majority of studies have mainly focused on the response of HSP70 *per se*

and as yet, the stress response of human muscle therefore remains to be accurately determined.

The following sections provide a critical review of exercise related HSP data (both acute and chronic) and attempts to identify some of the methodological factors that may be influencing the response. Although attention is primarily directed to human studies, findings from animal studies, which may be applicable to the exercising human, are also reviewed where appropriate.

2.4.1 Acute exercise response

2.4.1.1 Treadmill Protocols

Puntschart *et al.* (1996) were the first authors to provide data regarding the heat shock response following acute exercise in human muscle. These authors demonstrated that although HSP70 mRNA increased 4 fold in the vastus lateralis muscle immediately post 30 min of treadmill running at the anaerobic threshold (and remained elevated 3h post-exercise), HSP70 protein levels did not change within 3h after cessation of exercise. The authors attributed this finding to the fact that there may be a time delay between transcription and translation and that a 3h time period post exercise was not sufficient for newly synthesised protein to be detected. It was also speculated that the absence of an increase in HSP70 protein levels may have occurred due to high pre-existing protein levels and that a rather high amount of pre-existing HSP70 masked the appearance of newly synthesised protein.

The only other study to examine HSP70 expression in human muscle using treadmill exercise is the more recent research of Walsh *et al.* (2001) where five active males performed 60 min of treadmill running at an intensity corresponding to 70% $\dot{V}O_{2max}$. HSP72 content in the vastus lateralis was determined pre-exercise, immediately post and at 2, 8 and 24 h post-exercise. In contrast to Puntschart *et al.* (1996), HSP72 mRNA was not significantly increased immediately post-exercise yet did increase 6.5-fold 2 h post-exercise. This increase returned to basal levels and was not different from rest at 8 and 24 h post exercise. HSP72 protein levels did not significantly

change from rest at any time point although these data displayed substantial individual variation with 2 subjects showing marked increases in protein content at 8 h post-exercise.

The lack of data concerning the stress response of skeletal muscle to running exercise protocols is somewhat surprising given that running is the most popular form of 'keep fit' activity and is the main activity involved in an array of sports. At present, it is therefore difficult to conclude that running exercise is a sufficient stimulus to up-regulate HSP expression. Evidence from rodent muscle (Milne and Noble, 2002) suggests that the exercise protocols used in the aforementioned studies were not sufficient in terms of exercise intensity to initiate an overall increase in HSP content. Milne and Noble (2002) observed an exercise intensity dependent relationship of exercise-induced elevation of HSPs that appeared reflective of muscle recruitment patterns. For example, HSP70 displayed a significant increase in the soleus muscle following 60 min of treadmill running at low to moderate speeds only (15 to 27 m/min) whereas it exhibited a significant increase in the red and white portions of the vastus muscle only when exercise was performed at the highest running speeds (27 to 33 m/min). These data may therefore be of particular relevance for human studies in that a 'critical threshold' intensity may also be required to induce increases in muscle HSP content.

Alternatively, it may be that the timing of biopsy sampling in the previous studies was not appropriate to detect any newly synthesised HSPs, a view later supported by Khassaf *et al.* (2001). The above running protocols were indeed sufficient to induce an increase in HSP70 transcription suggesting that the appropriate signalling pathway had been activated. Exercise-induced translation of HSPs may therefore require a greater than 24 h period post-exercise and thus biopsy samples beyond 24 h post-exercise may be needed so as to detect increases in HSP content. Post-exercise biopsy sampling in the studies undertaken in the present thesis will therefore be extended beyond 24 h so as to provide a more accurate indicator of the time-course of the exercise-induced stress response of human muscle.

2.4.1.2 Cycling Protocols

Several authors have used cycling protocols as the exercise stimulus to initiate the stress response (Febbraio and Koukoulas 2000; Khassaf *et al.*, 2001, 2003; Jackson *et al.*, 2004). Febbraio and Koukoulas (2000) observed an approximate 2-fold increase in HSP72 mRNA in the vastus lateralis of 5 untrained male subjects immediately post exhaustive cycling exercise at 63% of $\dot{V}O_{2peak}$. Unfortunately, HSP72 protein levels were not measured due to insufficient tissue. More recently, Febbraio *et al.* (2004) failed to detect any increase in HSP60 and HSP72 content in active male subjects immediately post 2 h of semi-recumbant cycling at 65% $\dot{V}O_{2max}$. As discussed previously, however, it is likely that longer sampling periods following exercise are needed to allow translation of HSPs to occur as opposed to an inability of cycling exercise to initiate a stress response.

This has been most well documented by Khassaf and colleagues (2001). Using a 1-legged model at 70% of $\dot{V}O_{2max}$, these authors provided the first report of increased HSP content of human muscle of sedentary male subjects following acute exercise. HSP60 content of the vastus lateralis tended to increase 24 h post-exercise although this increase only became significant 3 days into the recovery period where protein content increased to 190% of pre-exercise values. HSP70 also tended to increase 1-2 days post-exercise although these values did not become significant until 6 days post-exercise where large dramatic increases were observed.

The pattern of HSP70 production, in accordance with previous research (Walsh *et al.*, 2001) also showed marked individual variation in terms of both time-course and magnitude of response (see Figure 2.2). This appeared to be due to a smaller proportionate response in those subjects in whom baseline HSP levels were relatively high. Typically, those individuals with relatively low levels of HSP70 responded to exercise with a faster (i.e. 24 – 48 h exercise) and larger increase in HSP70 expression whereas subjects with higher baseline levels exhibited a slower (i.e. 72 h – 6 days post-exercise) and much smaller response. Based on their time-course approach, these authors speculated that 48 h post-exercise was an appropriate time point to detect increases in HSP levels. Indeed, using this time point, Khassaf *et al.* (2003)

and Jackson *et al.* (2004) later confirmed the ability of the 1-legged model to induce a stress response in untrained subjects where 3-5-fold increases of HSP60, HSP70 and HSC70 were observed. Due to the large individual variability observed in their initial study (Khassaf *et al.*, 2001), however, the time-course of exercise-induced production of HSPs remains to be well defined.

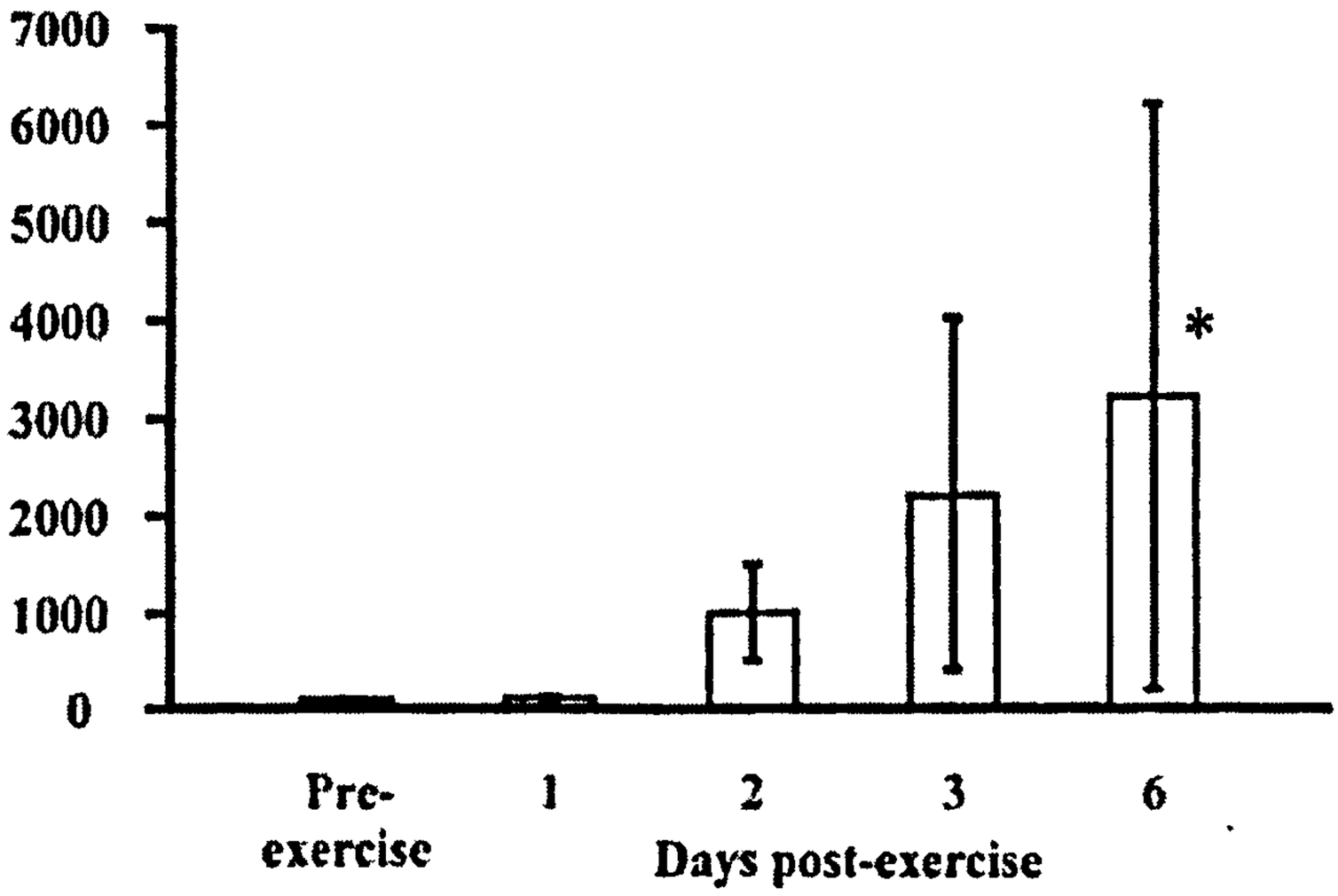

Figure 2.2 – HSP70 content in the vastus lateralis (expressed as a percentage of pre-exercise levels) pre- and post 45 min of 1-legged cycling exercise at 70% VO_{2max} (Khassaf *et al.*, 2001). Notice the large individual HSP response, possibly reflecting the large inter-subject differences in baseline HSP levels.

2.4.1.3 Resistance exercise and down-hill running protocols

A number of studies (Thompson *et al.*, 2001, 2002) have also investigated the HSP response to acute bouts of resistance exercise that are damaging in nature. In the first of these studies, HSP27 and HSC/HSP70 increased by 234 and 1064% respectively in the biceps brachii of untrained subjects 48 h after 2 sets of 25 repetitions of the eccentric portion of a biceps curl exercise. Thompson *et al.* (2002) later examined the

role of HSPs in the 'repeated bout effect' in which the previous resistance exercise protocol was performed on two occasions, separated by 4 weeks. Not unexpectedly, indirect indicators of muscle damage changed dramatically and significantly after the first bout of exercise but had a much smaller response after bout 2, thereby confirming the repeated bout effect. The relative magnitude of the increase in HSP27 and HSP70 response was the same after both bouts of exercise although the basal levels of both proteins in both the control and exercised samples of bout 2 were lower. Although the rates of synthesis of HSP27 and HSP70 were similar after both bouts, the absolute increase in protein levels was lower after bout 2. These data appear to represent an adaptive mechanism of the stress response whereby basal levels of stress proteins may be down regulated following repeated bouts of exercise but yet the magnitude of HSP expression following acute bouts of exercise is maintained or even increased. Although this hypothesis is speculative, several other authors have also reported data portraying a similar phenomenon in the skeletal muscle of trained rodents (Gonzalez *et al.*, 2000) and leukocytes of trained humans (Fehrenbach *et al.*, 2000) following an acute exercise stress and an *in vitro* heat shock, respectively. The effects of training status on basal HSP levels and the ability to mount a stress response is discussed in section 2.4.2.

Data are also available examining the stress response following downhill running protocols (Feasson *et al.* 2002; Thompson *et al.*, 2003). These protocols are also damaging in nature due to their bias towards lengthening contractions and are therefore discussed here as opposed to section 2.4.1.1. Feasson *et al.* (2002) subjected 12 untrained male subjects to 30 min of downhill running (12% gradient) at an intensity corresponding to 53.9 ± 1.5% of $\dot{V}O_{2max}$. The authors focused on the response of αB-crystallin and HSP27 due to the role of the small HSPs in the assembly and maintenance of the intermediate filament network (Mounier and Arrigo, 2002). A 2.8-fold and 2.2-fold of HSP27 and αB-crystallin was observed at 24 h post-exercise respectively. These increases were still evident at 14 days post-exercise, suggesting that the small HSPs play an integral role in the assembly/maintenance and remodelling of myofibrillar structures following exercise-induced muscle damage. The up-regulation of these proteins at 14 days post-exercise also suggests that they may play a protective role against further damaging stresses.

In contrast to the above data, Thompson *et al.* (2003) observed no increase in HSP27 or HSP70 levels 48 h following a downhill running protocol (10% gradient) at a mean heart rate of 77% of subjects' age predicted maximum. It is difficult to explain the discrepancies between studies although it should be noted that the subject base of Thompson *et al.* (2003) also included female subjects and thus may provide evidence of a gender specific stress response of human skeletal muscle to exercise. A gender specific response of HSP70 following an exercise stress is now well documented in animal models (Paroo *et al.*, 2002) where female rodents have displayed a diminished stress response to exercise. This issue is discussed further in section 2.4.1.5.4.

It is difficult to draw firm conclusions regarding the impact of the above described exercise protocols on muscle HSP production because of the inflammatory response that occurs in the days following damaging exercise. It is therefore possible that many of the reported increases in HSP content in these studies are also due to the increased presence of phagocytic cells given that such cells contain relatively high levels of HSPs (Khassaf *et al.*, 2003). The invasion of phagocytic cells would also result in neutrophil derived increases in free radicals thus contributing to secondary muscle injury that may further augment the intra-muscular expression of HSPs.

Analysis of muscle homogenates obtained from muscles that have undergone exercise protocols that are damaging in nature therefore make it extremely difficult to specifically quantify those HSPs that are induced by skeletal muscle gene expression by factors occurring during exercise (i.e. increased temperature etc) or are merely due to changes in phagocytic cell content and resulting secondary signalling pathways (Vasilaki *et al.*, 2006). Non-damaging exercise protocols (where non-damaging is defined as exercise that induces no overt structural or functional damage to the muscle), such as those discussed in section 2.4.1.1 – 2.4.1.2, have therefore been suggested to provide a more controlled and cleaner methodological approach by which to study the exercise-induced regulation of HSPs (Vasilaki *et al.*, 2006). A non-damaging exercise protocol will therefore be employed in all exercise related studies undertaken in the present thesis.

2.4.1.4 Time-course of the response

In light of the above data, it appears that the heat shock response in human tissue occurs with a somewhat greater time delay than that typically observed in animal models where an increase in HSP protein levels can be expected within several hours post-exercise. In humans, HSP mRNA has been shown to be increased during (Febbraio and Koukoulas, 2000), immediately (Puntschart *et al.*, 1996) or several hours post-exercise (Walsh *et al.*, 2001) whereas an increase in protein levels has only been observed 1-14 days post-exercise (Feasson *et al.*, 2002; Khassaf *et al.*, 2001,2003; Jackson *et al.*, 2004; Thompson *et al.*, 2001, 2002). Based on those studies that have performed serial biopsies either within one day (Walsh *et al.*, 2001) or on consecutive days (Khassaf *et al.*, 2001), it would appear that 48 h post-exercise is a reasonable time point at which to detect significant increases in HSP levels. This time point has indeed been adopted in many of those studies where only one post-exercise biopsy has been performed (Khassaf *et al.*, 2003; Jackson *et al.*, 2004; Thompson *et al.*, 2001, 2002, 2003). The time-course of the stress response, however, may be specific to particular HSPs and may also be dependent on the characteristics of the chosen exercise protocol (e.g. intensity / duration / mode / damaging / non-damaging). The response of some of the major HSP families outlined in section 2.3 will therefore be simultaneously examined in all experimental studies undertaken in this thesis.

2.4.1.5 Individual variation of the response

The exercise-induced stress response of human muscle appears to display remarkably high individual variation (in terms of both magnitude and time-course), which appears to be due, in part, to individual differences in baseline HSP levels (Walsh *et al.*, 2001; Khassaf *et al.*, 2001). In considering factors that determine basal HSP levels and / or the extent of the heat shock response to various stresses, training status (Gonzalez *et al.*, 2000), recent activity levels (Campisi *et al.*, 2003), thermal history (Kregel, 2002), energy availability (Febbraio *et al.*, 2002a), gender (Paroo *et al.*, 2002) and age (Vasilaki *et al.*, 2002) have all been considered as possible determinants. The roles of the above factors in influencing the magnitude of the exercise induced stress response

are discussed in the following sections. An understanding of the potential influence of these factors is extremely important when recruiting subjects for which to examine the exercise-induced stress response of human muscle.

2.4.1.5.1 Training status

It has been suggested that trained subjects may exhibit a diminished stress response to exercise of a customary nature, given that the adaptations that occur during exercise training function to reduce the degree of homeostatic unbalance that occurs during a given exercise stress (Smolka *et al.*, 2000; Nething *et al.*, 2004; Liu *et al.*, 2006). Alternatively, several data suggest that trained muscle may respond to an acute exercise stress with a faster and larger production of HSPs thereby helping the cell to recover quicker (Campisi *et al.*, 2003; Gonzalez *et al.*, 2000; Fehrenbach *et al.*, 2000). A more comprehensive discussion of these data is provided in section 2.4.2.2. Although it is presently unclear how training status influences the magnitude of the exercise-induced stress response of human skeletal muscle, it is nevertheless apparent that subtle differences in training status between subjects may contribute to the individual variation observed previously both within and between studies.

2.4.1.5.2 Recent activity levels / thermal history

The cellular content of HSPs at any given time may simply be a reflection of the previous exposure to acute periods of stress such as physical activity (i.e. exercise) or hyperthermia. Data suggest that some HSPs may indeed remain elevated for up to 14 days following an acute exercise stress (Feasson *et al.*, 2002). There is also evidence that heat acclimatised organisms display higher basal levels of HSPs in various cell types. For example, in a survey of lizard species inhabiting a variety of environments, the liver level of HSP70 was correlated to the level of the environmental niche (Ulmasov *et al.*, 1992). Skin fibroblasts from individuals living in a hot climate also contain higher HSP70 levels than those cells from individuals living in a moderate climate (Lyashko *et al.*, 1994). In a similar manner to that of trained muscle, those cells demonstrating elevated levels of HSPs following a recent stress may therefore

not need to exhibit a further production of HSPs following an acute exercise stress as they may already be pre-conditioned to such stresses. Variations in the basal levels of HSPs and in the magnitude of the exercise-induced production of HSPs may therefore be related to the timing of previous exposures to stress. Non-heat acclimatised and rested individuals should therefore be utilised in all of the experimental studies in the present thesis. Precisely how long, however, that is required to alleviate the acute expression of HSPs is not well defined.

2.4.1.5.3 Energy availability

Reduced glucose availability activates the stress response *in vitro* (Sciandra and Subjeck, 1983) and a reduction in muscle glycogen has also been suggested to be a contributing factor to the exercise-induced production of HSPs in human skeletal muscle (Febbraio and Koukoulas, 2000; Febbraio *et al.*, 2002b). Exactly how carbohydrate availability may regulate HSP expression is unclear and a more comprehensive discussion as to the role of energy availability in activating the stress response is provided in section 2.5.3. Nevertheless, these data suggest that inter-subject variations in pre-exercise muscle glycogen content may contribute to the variability of the exercise-induced induction of HSPs. It is extremely difficult, however, to ensure similar glycogen levels between subjects as resting muscle glycogen content is dependent upon recent activity, training status and predominant fibre type (Maughan *et al.*, 1997).

2.4.1.5.4 Gender

It is suggested that female rodents (Amelink and Bar, 1986) and humans (Shumate *et al.*, 1979) experience exercise-induced muscle damage to a lesser degree than that of their male counterparts. Females should therefore display a decreased intracellular accumulation of denatured proteins than males and thus it follows that females should exhibit an attenuated HSP response to exercise (Paroo *et al.*, 1999). In keeping with this hypothesis, Paroo *et al.* (1999) observed significantly lower post-exercise HSP70 content in female rodent heart, lung, liver and skeletal muscle when compared to male

rodents. Estrogen treated males also exhibited a blunted stress response to exercise, similar to that observed for female rodents. The same workers later demonstrated that removal of ovaries from female rodents resulted in muscle HSP induction similar to that of male rodents and further showed that estrogen treatment to ovariectomized animals reversed this effect (Paroo *et al.*, 2002). Although the exact mechanisms by which estrogen attenuates HSP70 induction with exercise are unclear, it is thought to be mediated through an indirect antioxidant activity and stabilization of cellular membranes (Paroo and Noble, 2002). A gender specific HSP response of human skeletal muscle to exercise may therefore explain some of the discrepancies between studies reviewed previously.

2.4.1.5.5 Age

A growing body of literature indicates that the stress response is severely attenuated with ageing. This is the case in a variety of cell types following a number of stressors. For example, tissues from aged animals (Kregel and Moseley, 1996; Zhang *et al.*, 2002) and lymphocytes (Jurivich *et al.*, 1997; Rao *et al.*, 1999) and monocytes (Njemini *et al.*, 2003) from elderly humans show a reduced production of HSPs following heat stress. Following an exercise related stress, the myocardium (Demirel *et al.*, 2003) and skeletal muscle (Vasilaki *et al.*, 2002) from aged rodents also exhibit a diminished HSP response when compared with their younger counterparts. The precise mechanisms underlying the attenuated stress response of aged skeletal muscle following contractile activity is an active area of research (Vasilaki *et al.*, 2006) and may be related to transcriptional defects. These data therefore suggest that future research should employ subjects from similar age ranges so as to eliminate the potential individual variability of the exercise-induced stress response.

2.4.1.6 Summary

Available data now demonstrate that acute forms of endurance and resistance exercise up-regulates several HSPs in human skeletal muscle. Despite these recent advances, the exercise-induced stress response remains poorly characterised and understood.

This is due to variations in timing of biopsy sampling between studies, restrictions of analysis to one or two HSPs *per se* (most notably HSP70) and differing subject characteristics both within and between studies. Future researchers should therefore focus their efforts in obtaining tissue from carefully chosen time points (with respect to their chosen exercise protocol) and also examine the response of several HSP families. The importance of utilising subjects from a relatively homogenous population (i.e. age, gender, training status, nutritional status, recent activity levels and thermal history etc) should also not be underestimated as all of these factors appear capable of influencing the magnitude of the stress response.

2.4.2 Exercise training

Whereas increased production of HSPs following an acute exercise stress primarily functions as an attempt to recover and regain cellular homeostasis, changes in HSP content that occur with repetitive exercise likely represent adaptations that serve to maintain homeostatic balance during a given stress (Noble, 2002). An up-regulation of basal HSP content during chronic exercise may therefore be a crucial component of the cellular and molecular mechanisms by which exercise confers protection to cells and tissues against a variety of related and non-related stressors (Locke, 1997). This concept of acquired cross-tolerance, where stressor A (e.g. exercise) confers protection against stressor B, is further discussed in section 2.6.1. An increase in basal HSP levels in trained muscle may also of be particular importance in facilitating the cellular remodelling processes that are inherent of the training response (see section 2.6.2).

A wealth of data suggests that various types of chronic contractile activity (e.g. chronic electrical stimulation, treadmill training) is accompanied by an increase in basal HSP levels in both cardiac (Demirel *et al.*, 1998; Powers *et al.*, 1998, 2001; Noble *et al.*, 1999; Samelan, 2000; Harris and Starnes, 2001; Atalay *et al.*, 2004) and skeletal muscle (Kelly *et al.*, 1996; Neufer *et al.*, 1996; Echochard *et al.*, 2000; Gonzalez *et al.*, 2000; Mattson *et al.*, 2000; Samelan, 2000; Samelan *et al.*, 2000; Atalay *et al.*, 2004). As highlighted earlier, a comprehensive review of the stress response of animal tissue is beyond the scope of the present review and thus the

reader is referred to several excellent reviews (Locke and Noble, 2002; McArdle and Jackson, 2002, Liu and Steinacker, 2001; Liu *et al.*, 2006).

The elevation of basal HSP levels in animal muscle has led to the question of whether training status blunts the ability to respond to additional or novel stresses with a further increase in HSP production (Noble, 2002). Alternatively, there are several reports that exercise training enhances the ability to respond to either novel or customary stresses (Gonzalez *et al.*, 2000; Campisi *et al.*, 2003). In contrast to animal muscle, data from human muscle concerning the influence of training status on both basal HSP levels and the ability to produce HSPs following an acute exposure to stress are extremely limited. These issues are discussed in the following sections.

2.4.2.1 Basal HSP content

Research from Liu and colleagues (1999, 2000, 2004) has presented data appearing to portray similar findings as to those observed in animal muscle. In their initial study (Liu *et al.*, 1999), 10 elite male rowers performed a 4-week training programme consisting of 4 distinct phases whereby exercise volume was different in each phase (1 week represented 1 phase). The maximum increase in HSP70 content of the vastus lateralis (123% over resting levels) was found at the end of the second phase of training when the maximum amount of exercise had been completed. With the exercise volume decreasing during the third and fourth weeks, the increase in HSP70 began to attenuate with HSP70 levels at the end of training declining towards resting values. These data suggest that the HSP response to training is related to the total exercise volume although at this stage the authors could not comment on whether such a response was dependent on the intensity or volume of the exercise stress.

In a subsequent study, Liu *et al.* (2000) attempted to address the relative importance of exercise intensity and volume in contributing to increased HSP synthesis. In this study, 14 elite male rowers were split into two groups (A, n =6; B, n = 8) and each group performed a training programme consisting of 3 distinct phases where exercise intensity was changed in each phase. Group A performed higher intensity training in phase 1 whereas group B performed higher intensity training in phase 2. Total

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training volume was matched in both groups during the first two phases. During the third phase, both volume and intensity was reduced in both groups. Muscle biopsies were again taken from the vastus lateralis prior to training and at the end of each training phase. The highest level of HSP70 in group A was observed at the end of training phase 1 while in group B it was observed at the end of phase 2. Reduction in training volume and intensity during phase 3 was associated with declines of HSP70 levels to basal levels in both groups (see Figure 2.3). These data suggest that the HSP70 response to training is most dependent on exercise intensity. This, of course, sounds physiologically reasonable given that high intensity exercise is associated with greater core temperature, muscle lactate, glycogen depletion and production of free radicals (see section 2.5 for a discussion of possible signals responsible for initiating the stress response).

Figure 2.3 – HSP70 response to training during 3 phases of different training intensity. Notice that in phase 3 when exercise intensity had decreased, HSP70 expression in both group A (open bars) and B (hatched bars) had returned to pre-training values (Liu *et al.*, 2000).

Importantly, the data of Liu *et al.* (2000) suggest that the increases in HSP levels observed with training are only maintained if the training stimulus (both intensity and volume) is either held constant or increased. If the training stimulus is reduced, HSP levels appear to return to pre-training baseline levels. These data are of important significance in that HSP mediated protection is also likely to return to pre-training values. It is therefore possible that a down-regulation of HSP content upon the cessation of an exercise training programme (or in a period of de-training) may be one of multiple mechanisms by which the repeated bout effect can be quickly attenuated.

The effects of different modes of exercise training on the HSP70 response in human muscle have also been recently studied (Liu *et al.*, 2004). Six elite male rowers underwent a training programme consisting of two 3 week phases separated by a 1 week recovery period. In the first phase, subjects performed high intensity strength training (blood lactate levels ranging from 4.7 to 11.3 $mmol.l^{-1}$) and in the second phase the subjects performed low intensity endurance training (blood lactate levels ranging from 1.3 to 3.0 $mmol.l^{-1}$). HSP70 content of the vastus lateralis increased significantly by 43% at the end of phase 1. It then decreased significantly during the 1 week recovery period and remained unchanged throughout the 3 week endurance training period. Nevertheless, the HSP70 content was still above pre-training levels at the end of the total training programme. With regard to the differential response to each training regime, it is possible that the apparent blunted HSP70 response to endurance training was due to the prior bouts of high intensity resistance training, which may have pre-conditioned the muscle for a subsequent stress. Indeed, HSP70 levels did remain above baseline throughout endurance training and may therefore not have needed to increase any further to combat the disruptions in homeostasis caused by low intensity exercise. Alternatively, considering all subjects were elite athletes and the low intensity nature of this particular training phase, it may simply be that this period of exercise did not induce strong enough stimuli to mediate a stress response. This is contrast to resistance exercise where a large and dramatic heat shock response is usually observed (Thompson *et al.*, 2001, 2002). These data support those issues discussed previously whereby the exercise-induced production of HSPs during both acute and repeated exercise appears related to exercise intensity, individual training status and recent activity.

Although the above studies have provided the initial data regarding the stress response to exercise training, they are limited in their design and their failure to disclose the precise time point of biopsy sampling in relation to the last training session. This is particularly important considering that in some individuals, HSP70 may take 3 - 6 days to increase above resting levels in response to an acute bout of exercise (Khassaf *et al.*, 2001). Given that the HSP response is generally regarded as a transient response required by cells to allow them to adapt to a new level of stress (Locke, 1997) it is possible that many of the reported increases in HSP levels in the previous studies and animal literature simply reflect the change in exercise intensity/duration from the previous exercise session. Furthermore, the subjects used in the previous investigations were already of elite nature and thus it is possible that the short-term training interventions were insufficient to induce further gross changes in muscle HSP levels. As such, the pre-training intervention levels of HSPs in these subjects may have already been appropriate to counteract the stress of customary training routines. It is therefore possible that HSP levels of these subjects are somewhat higher than those of less conditioned or sedentary population.

It would therefore seem appropriate to also employ additional experimental designs that allow the impact of training on basal HSP levels to be further characterised. Such designs may include cross-sectional studies whereby the basal levels of HSPs in both trained and untrained individuals who are in a fully rested state could be readily compared. This may provide a more accurate representation of those HSPs induced by chronic exercise as opposed to those induced as a transient response from the last bout of exercise undertaken. Precisely how long, however, that is required to alleviate the acutely produced HSPs remains to be defined. The time-course of reduction of HSP levels to pre-training values following the cessation of training also remains to be characterised.

2.4.2.2 Magnitude of the stress response

In addition to adaptations of basal HSP status following exercise training, it is also important to consider how training affects the ability to synthesise HSPs following an acute stress. It has been suggested that exercise training might allow an individual to

mount a greater and/or faster stress response following an acute bout of stress (Gonzalez *et al.* 2000). These authors observed that when trained rodents performed an acute bout of treadmill running (of lower intensity than the customary training sessions) 3 days after their last training session, the ratio of their post-exercise vs resting synthetic rates of HSP72 was significantly elevated compared to sedentary controls. Similar observations have been made from blood leukocytes of trained humans where the application of an *in vitro* heat shock to resting leukocytes of trained and untrained individuals induced a significantly higher increase in HSP70 gene expression in trained subjects (Fehrenbach *et al.*, 2000). Consistent with these findings, Campisi *et al.* (2003) also demonstrated that habitual physical activity facilitates stress-induction of HSP72 in brain, peripheral and immune tissues. These authors showed that rodents who had previously completed 8 weeks of voluntary free wheel running exhibited a larger increase in HSP72 than sedentary rats in brain, heart, liver and spleen tissue following a novel tail shock stress or exhaustive treadmill running exercise. Taken together, the above data suggest that trained individuals may have an increased ability to recover and regain cellular homeostasis following an acute bout of novel or customary stress via the increased ability to synthesise HSPs. It is presently unclear, however, if this mechanism is in operation in the skeletal muscle of humans following an acute exercise stress of either customary or novel nature.

Alternatively, it is possible that the HSP response to exercise in the trained state is associated with a reduced stress response as the muscle is already pre-conditioned with appropriate endogenous defence systems to combat any homeostatic disruptions. Several data also appear to support this hypothesis. For example, trained rodents display a blunted production of HSP70 in soleus muscle than sedentary rodents following an acute exercise protocol matched for both absolute treadmill speed and duration (Smolka *et al.*, 2000). This finding was attributed to a training-induced increase in baseline protective systems such as increased activity of antioxidant enzymes of catalase and glutathione reductase. It was therefore suggested that HSPs act as a secondary antioxidant defence system providing additional protection when the primary system is overwhelmed. In a preliminary finding from humans, Nething *et al.* (2004) observed that a group of well trained rowers did not show any increase in muscle HSP70 gene and protein expression within 6 hours following a high intensity

strength training session or low intensity endurance training session. These data are limited, however, in that a less-conditioned control rowing group were not studied. Further studies where both trained and untrained groups are compared in their stress response to an acute exercise stress are therefore necessary. Such investigations should also obtain biopsies beyond the time-point of 6 h post-exercise so as to examine changes in HSP content.

2.4.2.3 Summary

It is clear that our current understanding of how periods of exercise training affect both basal HSP levels and also the ability to mount a stress response following an exercise stress is extremely limited. This is particularly the case in relation to the exercising human. Available data indicate that training up-regulates basal HSP levels in a complex manor that is dependent on exercise intensity / duration and also the individual's initial training status. The magnitude of the stress response following acute exercise in trained muscle is also not well defined although preliminary reports suggest that trained muscle exhibits a blunted production of HSPs to customary exercise. These questions bear important significance from both a health and performance perspective and will therefore be addressed in this thesis.

2.5 Possible Physiological Signals Initiating The Exercise-Induced Stress Response

The cellular stress response has been shown to be activated both *in vitro* and *in vivo* following various types of stress including thermal, oxidative, mechanical, metabolic and cytokine production. These stressors are similar to the homeostatic perturbations occurring in contracting skeletal muscle and thus it is difficult to isolate the precise signal(s) that is responsible for initiating the exercise-induced stress response. In the present section, data concerning the role of the above factors in contributing to the exercise-induced stress response is discussed. Given the limited amount of data from human studies that have addressed this issue, data from animal studies will also be discussed.

2.5.1 Temperature

A major portion of the energy utilised by skeletal muscle to perform physical work is converted to thermal energy, ultimately translating to an increase in temperature of the contracting muscle and in whole body core temperature. Given that heat shock was the first stimulus discovered to induce HSP expression (Ritossa, 1962; Tissieres *et al.*, 1974) and also the role of HSPs in providing thermotolerance (see section 2.6.1), it is therefore tempting to speculate that exercise-induced hyperthermia is the dominant signal that is responsible for protein denaturation and the subsequent initiation of the cellular stress response during exercise.

Exercise-induced increases in core and contracting muscle temperature are indeed routinely suggested as a possible factor for inducing an up-regulation of HSPs following exercise (Fehrenbach and Niess, 1999; Lancaster and Febbraio, 2005; Liu and Steinacker, 2001; Liu *et al.*, 2006). Supporting evidence for this hypothesis is provided from a number of cell types and experimental approaches. Although less thermally sensitive than cardiac tissue (Ali *et al.*, 1997), there are several studies demonstrating that heat shock to physiological temperatures to those comparable to exercise (Brooks *et al.*, 1971) can induce increases in HSP content of skeletal muscle.

For example, both *in vitro* (Maglara *et al.*, 2003) and *in vivo* (Oishi *et al.*, 2002, 2003) heating protocols in which cell temperature was increased to 42 °C induce increases in HSP70 content of C_2C_{12} skeletal muscle myotubes and rodent muscle, respectively. Kim *et al.* (2004) also demonstrated that the exercise-induced increase of HSP70 in the myocardium and soleus muscle during exhaustive treadmill running is significantly enhanced when exercise is performed under elevated ambient temperatures (41 vs 23 °C), despite reduced running times. Furthermore, when the exercise protocol was performed in cool ambient conditions (12°C), no increase in HSP70 content was seen in either tissue. It was therefore concluded that the exercise-induced accumulation of HSP70 is dependent on the absolute body temperature, irrespective of exercise duration. Elevations in cellular temperature *per se* can also induce increases in reactive oxygen species (Salo *et al.*, 1991; Zuo *et al.*, 2000) and carbohydrate utilisation (Febbraio, 2000), also thought to be potent activators of the exercise-induced stress response (see section 2.5.2 - 2.5.3). These data suggest that elevated temperature may influence HSP expression via a direct thermal modification of intracellular proteins and also as a result of secondary signalling pathways.

In contrast to the above, numerous data suggest that rises in core and muscle temperature may not be the sole signal responsible for the exercise-induced expression of HSPs. Skidmore *et al.* (1995) observed increases in HSP70 content in both skeletal and cardiac muscle of rodents 30 min after 1 h of treadmill running which were independent of an increase in core temperature. It is important to note, however, that an absence of a rise in core temperature does not necessarily mean that the temperature of the contracting muscle was stable. Indeed, Febbraio *et al.* (1996) blunted the rise in rectal temperature in humans during exercise in a cool environment and still observed a rise of 3.3 °C in the vastus lateralis muscle. Nevertheless, electrical stimulation of both rabbit (Neufer *et al.*, 1996) and rodent (McArdle *et al.*, 2001) muscle has induced increases in muscle HSP content in the absence of an increase in muscle temperature. The magnitude of HSP increases observed in animal studies following treadmill running (Locke *et al.*, 1990; Salo *et al.*, 1991; Skidmore *et al.*, 1995; Hernando *et al.*, 1997; Milne and Noble, 2002) or electrical stimulation protocols (Neufer *et al.*, 1996; McArdle *et al.*, 2001) is also somewhat larger than that observed following *in vivo* heating protocols (Oishi *et al.*, 2002, 2003) thus

suggesting that additional signals arising during exercise other than elevated temperature are contributing to the heat shock response. Based on available data, however, it is extremely difficult to ascertain the role of increased temperature as the primary activator of the exercise-induced stress response of human skeletal muscle *in vivo*. Despite a decade of research, this hypothesis remains to be formally tested and will therefore be investigated in this thesis.

2.5.2 Oxidative stress

A large body of evidence now exists suggesting that exercise-induced production of reactive oxygen species (ROS) and the associated modification of protein structure may be an extremely important signalling pathway up-regulating HSP expression. Free radicals and reactive oxygen species have long been considered as 'injurious molecules' because of their capacity to promote cellular damage and their involvement in the pathogenesis of a variety of diseases (Hamilton and Powers, 2002). However, evidence is now also emerging indicating that exercise-induced increases in ROS can act as important cellular messengers in signal transduction and activation of many redox regulated transcription factors such as NF-kB, activator protein 1 (AP-1) and HSF1 (Pattwell and Jackson, 2004, Jackson, 2005).

There are many potential sources of ROS within the cell including the mitochondrial respiratory chain, xanthine oxidase production of the superoxide radical, plasma membrane located systems for superoxide and nitric oxide production and neutrophil-induced oxidative bursts. A proposed scheme for the generation of ROS in skeletal muscle is shown in Figure 2.4. Although the relative contribution of each of these pathways during exercise remains an ongoing and active area of research, it is largely considered that the mitochondria may be the major site of ROS generation, especially during aerobic and endurance type exercise (McArdle *et al.*, 2002; McArdle and Jackson, 2002). Despite the fact that oxygen consumption during these type of activites is relatively high as is the tendency to form ROS, these form of contractions result in considerably less muscle damage than that induced by lengthening contractions (McArdle *et al.*, 2002). In these instances, it is suggested that exercise-induced increases in ROS production may therefore act as a signal for the activation

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of the stress response whereby the increased expression of HSPs provides protection against further insults and is involved in any necessary cellular remodelling rather than initiating damage (McArdle and Jackson, 2000).

McArdle *et al.* (2001) demonstrated that a 15 min period of mild non-damaging isometric contractions of the hindlimbs of mice results in increased release of superoxide anions from within the muscle into the extracellular space. This increased production of free radicals was accompanied by a transient oxidation of muscle protein thiols which was reversed within 1-2 hrs following the contraction protocol.

Despite the oxidation of muscle proteins, no evidence of overt cellular damage was seen, as demonstrated by circulating creatine kinase levels. The contraction protocol also resulted in a rapid and substantial increase in HSP production in the soleus (HSP60 and HSP70) and extensor digitorum longus (HSP70) muscles. These findings appear consistent with data from other cell types (Freeman *et al.*, 1995; McDuffee *et al.*, 1997) in that oxidation of muscle protein thiols may be part of a key signalling mechanism leading to increased HSP production. Comparable data have also been obtained from humans where an acute bout of exercise that resulted in increased total superoxide dismutase activity also induced an up-regulation of HSP60 and HSP70 content of the vastus lateralis (Khassaf *et al.*, 2001). These data suggest that subtle moments of redox imbalance may act as a signal for adaptation either by direct interaction with HSF1 (Ahn and Thiele, 2003) or by causing some minor oxidative damage to proteins that is detected by the cell subsequently leading to an up-regulation of HSPs.

Given the evidence that ROS can serve as a stimulus for HSP production, it follows that enhancing cellular antioxidant defences could inhibit HSP transcription thereby attenuating the accumulation of HSPs (Hamilton and Powers, 2002). This hypothesis has been confirmed following stressors such as heat shock (Gorman *et al.*, 1999), hypoxia (Borger and Essig, 1998), ischemia-reperfusion (Nishizawa *et al.*, 1999) and exercise (Hamilton *et al.*, 2003). These findings have recently been extended by a series of human studies demonstrating that the increased production of HSP70 content in human skeletal muscle following 1-legged cycle ergometry (Khassaf *et al.*, 2001) was effectively abolished following vitamin C (Khassaf *et al.*, 2003), vitamin E (Jackson *et al.*, 2004) or β-carotene supplementation (Jackson *et al.*, 2004). It should be noted, however, that antioxidant supplementation in these studies induced a significant increase in baseline muscle HSP70 content suggesting that an attenuation of the exercise-induced response may be related to an increase in baseline protective systems. Alternatively, it may be that an elevation of tissue antioxidant capacity directly scavenges exercise generated ROS thereby abolishing transcriptional activity of the HSP genes. Experimental support for this hypothesis was recently provided by Fischer *et al.* (2006) where the combination of vitamin C and E supplementation

inhibited increases in muscle HSP72 mRNA expression immediately following 3 h of knee extensor exercise.

The above studies demonstrate, unequivocally, a role of ROS in activating the stress response. A proposed simplistic relationship between ROS, antioxidants and HSPs is displayed in Figure 2.5. Further research is required to add to the depth of this relationship and alleviate the precise mechanisms underlying this model. This is likely to be a long process, however, as the precise source and species of exercise-induced radical production in skeletal muscle remains to be defined. This is particularly the case in humans where data concerning the exercise-induced oxidant production are extremely limited.

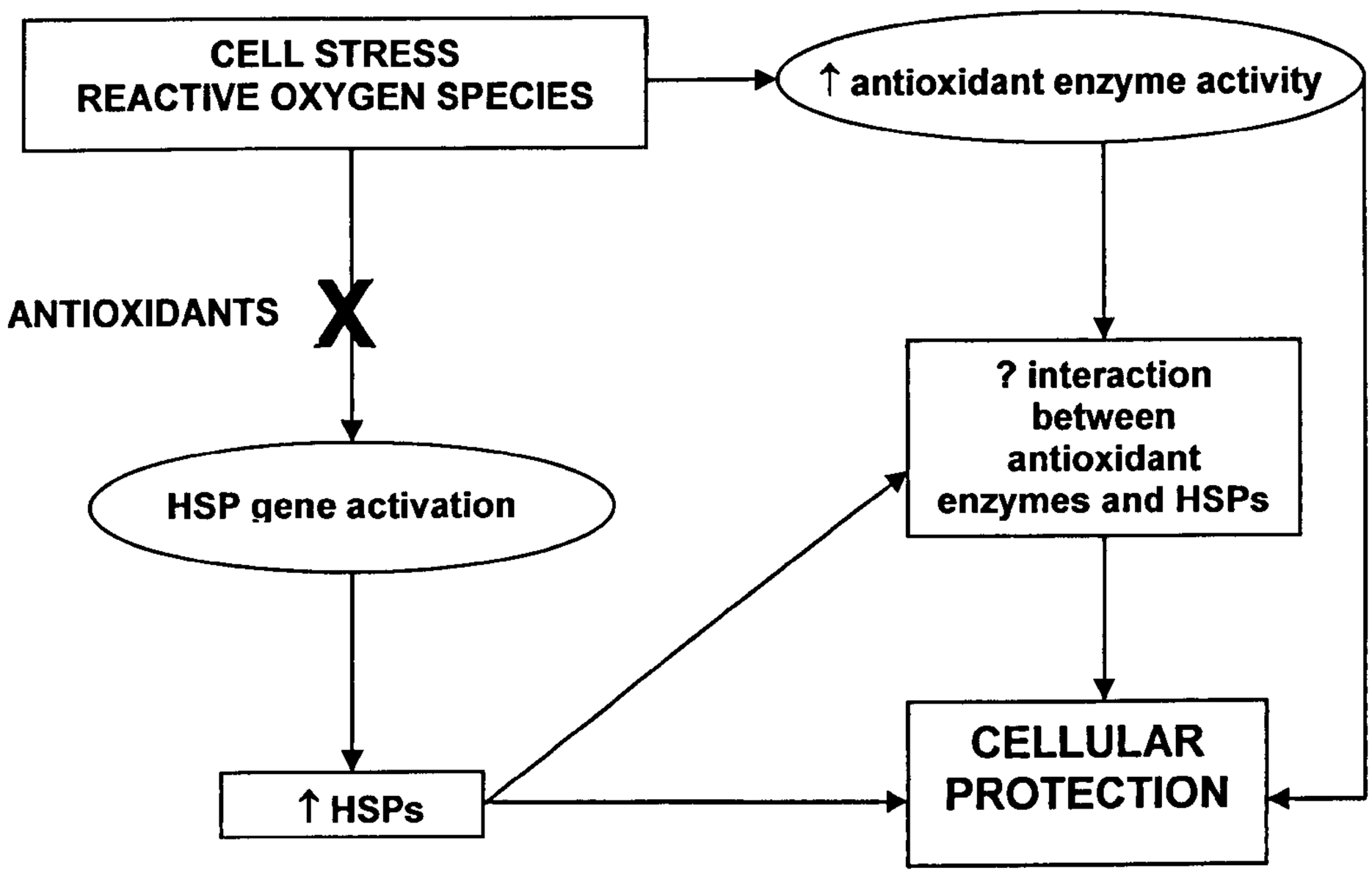

Figure 2.5 – Proposed relationship between HSPs, ROS and antioxidants (Hamilton and Powers, 2002). Cell stress (including ROS mediated stress) leads to up-regulation of HSPs and antioxidant enzyme activity ultimately leading to protection against subsequent cell stress. Exogenous antioxidants may interfere with activation of HSP genes, thus inhibiting HSP synthesis and related cell protection. HSPs may or may not exert their cellular protective effects in concert with antioxidant enzymes.

2.5.3 Metabolic stress

Reduced glucose availability (Sciandra and Subjeck, 1983), ATP depletion (Benjamin *et al.*, 1992), acidosis (Weitzel *et al.*, 1985) and increased intracellular calcium levels (Welch *et al.*, 1983) can all induce an increased production of HSPs. It is therefore unsurprising that exercise-related metabolic stress has also been suggested to be an important factor in initiating the exercise-induced stress response (Fehrenbach and Niess, 1999; Liu and Steinacker, 2001).

In myogenic cultured cells, ATP depletion to 30% of control levels and a fall in pH from 7.3 to 6.9 was sufficient to induce HSF1 activation (Benjamin *et al.*, 1992). Reductions in pH to 6.7 in the face of maintained ATP levels failed to activate HSF 1 (Benjamin *et al.*, 1992). These data suggest that ATP depletion, independent of acidosis and lactate accumulation, may therefore be an important metabolic pathway for the expression of HSPs during exercise. When ATP is reduced to a critical level, HSPs remain complexed to unfolded proteins and cannot be recycled. Consequently, the pool of free or available levels of pre-existing HSPs decreases thereby leading to trimerization of HSF1 and transcription of the HSP genes. It is difficult to ascertain the contribution of ATP depletion *per se* during whole body exercise, however, as ATP levels are rarely depleted to less than 60% of pre-levels, even when exercise is exhaustive (Bangsbo, 2000).

A series of studies in humans have indicated that availability of carbohydrate for the active muscles may be particularly important in contributing to the exercise-induced expression of HSPs in both skeletal muscle and the circulation. These data are consistent with observations that muscle glycogen depletion during exercise results in intracellular changes in the ATP/ADP ratio (Sahlin *et al.*, 1990; Spencer *et al.*, 1991; Baldwin *et al.*, 1999). As outlined above, this may thereby increase polypeptide bound HSP72, decrease free HSP72 levels and thus ultimately increase HSP72 expression. Febbraio and Koukoulas (2000) demonstrated that the observed increase in HSP72 gene expression during exhaustive cycling only occurred late during exercise when intramuscular glycogen levels were reduced to low levels (< 100 mmol kg dry wt^{-1}). Febbraio *et al.* (2002b) subsequently demonstrated that the magnitude of the exercise-induced stress response may, in part, be influenced by pre-exercise

muscle glycogen content. Using a 2-legged exhaustive knee extensor protocol, these authors observed a 2-fold increase in both HSP72 gene and protein expression immediately post-exercise only in the leg that performed a glycogen depleting protocol 24 h prior to the exercise bout. These data are limited, however, in that it may have been the preceding glycogen depleting protocol that was responsible for the increased HSP expression as opposed to the reduced pre-exercise glycogen levels. This would appear consistent with the time-course of the HSP70 response previously observed in certain individuals following an exercise stress (Khassaf *et al.*, 2001). Furthermore, whilst these data indicate that carbohydrate availability may be a contributing factor to the exercise-induced expression of HSPs, they do not provide definitive evidence for energy depletion as the primary signalling pathway.

2.5.4 Cytokine production and inflammatory stress

Several cytokines have previously been demonstrated to be positive regulators of HSP expression (Arrigo *et al.*, 1987; Ciocca *et al.*, 1993; Stephanou *et al.*, 1997), and thus it is unsurprising that cytokines produced during exercise has also been suggested as a possible inducer of the exercise-induced stress response (Fehrenbach and Niess, 1999; Liu and Steinacker, 2001). Although there is a moderate increase in the systemic concentrations of several cytokines, the appearance of interleukin–6 (IL-6) in the circulation is by far the most pronounced and precedes that of any other cytokine (Febbraio and Pedersen, 2002). The main sources of IL-6 production *in vivo* are stimulated monocytes/macrophages, fibroblasts and vascular endothelial cells (Akira *et al.*, 1993). The invasion of monocytes into the muscle and the associated production of cytokines may therefore be responsible, in part, for the exercise-induced expression of HSPs especially following the damaging exercise protocols reviewed in section 2.4.1.3 (Thompson *et al.*, 2001, 2002, 2003). In these circumstances, the controlled and deliberate generation of ROS by phagocytic cells may have also contributed to the intramuscular expression of HSPs.

It was commonly thought that the cytokine response to exercise was a consequence of an immune response resulting from exercise-induced muscle damage and that immune cells were therefore responsible for the exercise–induced increase in plasma IL-6

(Nieman *et al.*, 1998). Recent data, however, have indicated that the exercise-induced increase in plasma IL-6 levels occurs in the absence of overt muscle damage (Starkie *et al.*, 2000, 2001; Febbraio *et al.*, 2003) and that circulating monocytes are not responsible for the increase in plasma IL-6 levels observed post-exercise (Starkie *et al.*, 2000). It is now well-documented that IL-6 is produced locally in contracting skeletal muscle (Steensberg *et al.*, 2000; Febbraio and Pedersen, 2002; Pedersen *et al.*, 2001) and that its release into the circulation is largely responsible for the exercise-induced increase in plasma IL-6 levels (Steensberg *et al.*, 2000). In addition to its proposed role as a hormone regulating substrate metabolism (Febbraio and Pedersen, 2002; Pedersen *et al.*, 2001), it has also been suggested that muscle-derived IL-6 may act as a signalling molecule that is involved in the regulation of various other cellular processes including activation of the cellular stress response (Febbraio *et al.*, 2002a).

Febbraio *et al.* (2002a) demonstrated that the infusion of IL-6 into resting human skeletal muscle induced a significant increase in HSP72 gene expression *in vivo*. It was therefore suggested that elevations in muscle IL-6 production during muscle contraction may serve as an upstream signal for a stress response. These findings are based, however, on resting skeletal muscle and hence are not representative of contracting skeletal muscle where a further array of alterations in cellular homeostasis is occurring. Precisely how IL-6 may regulate HSP expression during exercise also remains to be determined. In line with cell culture studies (Stephanou *et al.*, 1997, 1998), Febbraio and colleagues (2002a) suggested that IL-6 may activate HSP expression via activation of signal transducer and activator of transcription 3 (STAT3).

Despite the above observation, experimental evidence against a role of IL-6 was provided by an antioxidant supplementation study in which the increase in HSP72 gene expression in the vastus lateralis immediately following 3 h of 2-legged knee extensor exercise at 50% of maximal power output was abolished following vitamin C and E supplementation, whilst having no effect on IL-6 gene expression (Fischer *et al.*, 2002). These data suggest that cytokine production, at least that of IL-6, is unlikely to be the dominant signal for activation of the exercise-induced stress response, particularly during non-damaging aerobic type activites.

2.5.5 Summary

It is difficult to conclude that exercise induces HSP expression through some unique pathway. Given the array of 'cross-talk' that occurs between signalling pathways in skeletal muscle (e.g. temperature, ROS formation and carbohydrate depletion etc), it may be that two or more factors are acting in concert with each other in contributing to the stress response. Furthermore, the extent to which each of the above stressors contributes to the exercise-induced expression of HSPs is likely dependent on the mode, intensity, duration and contractile nature of the exercise stimulus (see Figure 2.6). Following resistance or damaging exercise, the expression of HSP is likely initiated by mechanically induced damage to muscle proteins. In these cases, the expression of HSPs may be further augmented in the days post-exercise due to the inflammatory response and the associated production of ROS. Following endurance and aerobic type activities, expression of HSPs may be initiated by an increase in muscle temperature and/or exercise-induced redox signalling. In such circumstances, heat induced radicals may also contribute to the overall oxidant activity of the cell. Reductions in carbohydrate availability may also contribute to the production of HSPs, especially if the exercise is prolonged and is performed in elevated ambient temperatures.

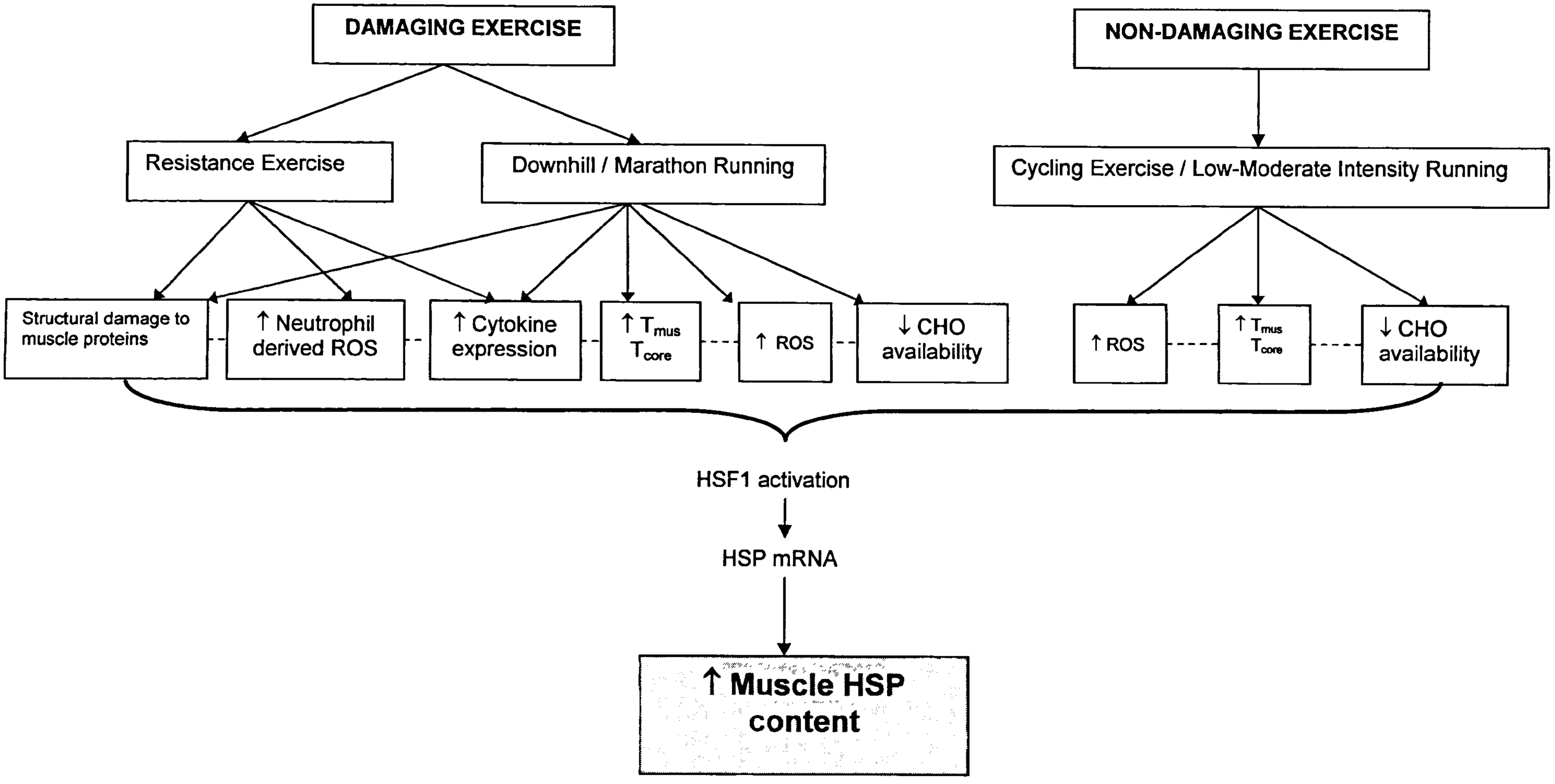

Figure 2.6 – Possible physiological signals initiating the heat shock response in human skeletal muscle during various forms of damaging and non-damaging exercise. Notice the 'cross-talk' between signals; it is possible that two or more signals are acting in concert with each other as opposed to one unique pathway. T_{mus}, muscle temperature; T_{core}, core temperature; ROS, reactive oxygen species.

2.6 Biological Significance of Exercise-Induced HSP Expression

The generic functions of HSPs as molecular chaperones and in providing cytoprotection were discussed briefly in section 2.2. In the following sections, these functions are reviewed in more detail with specific relation to the possible biological roles of the exercise-induced production of HSPs.

2.6.1 Acquired thermotolerance, cross-tolerance and cytoprotection

One of the first implied physiological functions of increased HSP expression was the phenomenon of acquired thermotolerance in which cells that were subjected to a sub-lethal heat shock were subsequently protected against normally lethal levels of heat shock (Landry and Chretin, 1983; Landry *et al.*, 1982; Li and Werb, 1982; Li *et al.*, 1983; Mizzen and Welch, 1988). These initial studies were generally correlative in nature with no causal link established between induction of HSPs and acquired thermotolerance. However, more advanced studies involving cellular manipulations that either block HSP accumulation or over-express certain HSPs provided direct evidence for the involvement of HSPs as cytoprotective proteins against heat stress (Angeledis *et al.*, 1991; Johnston and Kucey, 1988; Li *et al.*, 1991; Riabowol *et al.*, 1988).

The concept of acquired thermotolerance led to the logical suggestion of cross-tolerance in which cells that display elevated levels of HSPs following stressor A (which in most cases has been heat stress) are subsequently protected against a non-related stressor B (Li, 1983). Cross-tolerance has important implications for exercise whereby the exercise-induced production of HSPs may be an important mechanism by which exercise can provide protection to cells, tissues and organs against a variety of stressors and diseases (Locke and Noble, 2002). HSP mediated protection against a number of stressors is indeed now well documented where a variety of approaches have been employed to up-regulate pre-stress HSP content.

For example, with the use of transgenic models (Marber *et al.*, 1995) or pre-conditioning stresses such as hyperthermia (Currie *et al.*, 1988, 1993; Hutter *et al.*, 1994) and exercise (Locke *et al.*, 1995; Demirel *et al.*, 1998; Powers *et al.*, 1998, 2001), a large body of evidence demonstrates a role of HSPs (especially HSP70) in providing myocardial protection from ischemia-reperfusion injury (I-R). Exercise training can elevate HSP72 approximately 5-fold in the rat myocardium (Powers *et al.*, 1998) and thus the exercise-induced expression of HSPs is now widely considered as one of multiple mechanisms by which exercise can provide protection to cardiac tissue from damage (Taylor and Starnes, 2003; Starnes, 2002; Powers *et al.*, 2004).

Evidence for a protective role of HSPs against a number of damaging stressors in skeletal muscle is also emerging. Studies in rodents demonstrated that a prior heat stress (which resulted in increased muscle content of HSP70) provided significant protection to mature skeletal muscle against necrosis induced by ischemia-reperfusion (Garramone *et al.*, 1994; Lepore *et al.*, 2000). Maglara *et al.* (2003) also demonstrated that damage to C_2C_{12} mouse skeletal muscle myotubes induced by either the calcium inophore A23187 or the mitochondrial uncoupler 2,4-dinitrophenol (DNP) was significantly reduced by a prior period of hyperthermia, which induced significant increases in HSP25, HSC70 and HSP70 content. It was therefore suggested that an increased cellular content of HSPs may provide protection against the muscle damage that occurs by a pathological increase in intracellular calcium or uncoupling of the mitochondrial respiratory chain (i.e. exercise).

The protective effect of HSPs to skeletal muscle can also be mediated via the exercise-induced production of HSPs (F.McArdle *et al.*, 2004). In this study, hindlimb muscles of mice were subjected to a non-damaging contraction protocol *in vivo*. At 4 and 12 h following contractions muscles were subsequently removed and subjected to a damaging contraction protocol *in vitro*. The prior period of non-damaging stress was demonstrated to significantly protect the EDL and soleus muscles against the normally damaging *in vitro* protocol 4 h later. Workers from the same laboratory have also demonstrated that skeletal muscle of both adult and aged transgenic mice overexpressing HSP70 display enhanced recovery from damage induced by lengthening contractions when compared to wild type animals (A.McArdle *et al.*, 2004). Considering that exercise training can also up-regulate

basal HSP70 content of skeletal muscle (as reviewed in section 2.4.2), it follows that the exercise-induced expression of HSPs may therefore be a possible mechanism by which regular exercise provides protection to exercise-induced damage. Causative evidence for this hypothesis is provided from a training study in humans whereby the reduced creatine kinase levels during the progression of training was correlated with increased muscle HSP70 levels (Liu *et al.*, 1999).

2.6.2 Cellular remodelling, protein turnover and muscular adaptation

Acute and chronic exercise promotes an array of cellular adaptations which serve to reduce the homeostatic imbalance that occurs during a given exercise stress. This cellular remodelling process results from increased protein turnover arising from a delicate balance between protein synthesis and protein degradation. Depending on the nature of the exercise stimulus, adaptations such as muscle hypertrophy or mitochondrial biogenesis can occur. Considering the chaperone properties of HSPs, it is reasonable to assume that an increased cellular content of HSPs play an active role in facilitating the exercise-induced adaptations of skeletal muscle. This is discussed in the following sections.

2.6.2.1 Protein repair

Exercise creates an undesirable cellular environment for proteins and they are subject to attack by oxidation, increased temperature or metabolic acidosis (Goto and Radak, 2005). Proteins can therefore quickly lose their biological activities, gain harmful function and cause irreversible damage to the cell. The increased content of HSPs following an acute exercise stress is therefore likely to initially function in regaining intracellular protein homeostasis by refolding and reactivating misfolded or denatured proteins (Kilgore *et al.*, 1998). This function is thought to be particularly important in restoring post-exercise enzyme function (Kilgore *et al.*, 1998) and is also likely relevant to an unknown number of regulatory and structural proteins. The activity of several enzymes such as phospholipase A2 (Jatella , 1993), protein kinase C (Ritz *et al.*, 1993), lactate dehydrogenase (Zietara *et al.*, 1995) and citrate synthase (Locke *et al.*, 1994) have all been related to HSP70 concentration. HSP70 can also bind to the

fast twitch skeletal muscle sarcoplasmic reticulum Ca^{2+} ATPase and protect its function during thermal stress (Tupling *et al.*, 2004). Given the potential role of exercise-induced disturbances in activity of this protein pump (Holloway *et al.*, 2005) and an ensuing disturbance in calcium homeostasis as a cause of fatigue and damage (Gissel, 2005), it is possible that the acute exercise-induced production of HSPs also function to repair this enzyme's activity. In this way, the chronic induced elevations of HSPs during training may therefore reduce exercise-induced muscle damage that is mediated by calcium signalling pathways. The structural proteins are particularly susceptible to exercise-induced muscle damage / modification especially when the exercise is unaccustomed or involves lengthening contractions. The observation of increased HSP27 and HSP70 following lengthening contractions in both humans (Thompson *et al.*, 2001, 2002) and rodents (Koh and Escobedo, 2004) is therefore thought to aid in repair of the cytoskeletal proteins which may have been damaged during contractions.

2.6.2.2 Protein synthesis

Exercise results in the accumulation of specific proteins that ultimately serve to enhance homeostatic balance during a given exercise challenge. In this regard, it is reasonable to assume that the exercise-induced production of HSPs plays an important role in chaperoning these newly synthesised proteins to their correct structure and intended site of cellular action. Acute exercise causes a shift of HSPs toward 'affected' proteins and away from their normal chaperone role in unstressed situations (Thomason and Menon, 2002). The translocation of HSPs among intracellular pools and away from nascent polypeptides thereby decreases the polypeptide elongation rate (Ku *et al.*, 1995; Ku and Thomason, 1994). An increased production of HSPs following acute exercise may therefore simultaneously function to renature damaged proteins whilst attempting to maintain the required rate of protein synthesis (Thomason and Menon, 2002). The training induced increases in muscle HSP content may therefore have a profound effect on facilitating increased protein synthesis during training (Thomason and Menon, 2002).

2.6.2.3 Protein degradation

An exercise-induced production of damaged proteins and their subsequent accumulation can be potentially harmful to cells. It is therefore essential that damaged proteins are efficiently degraded and resynthesised so as to maintain normal cellular function (Reid, 2005). The process of protein degradation is therefore considered as equally important as transcription and translation (Goto and Radak, 2005). Degradation of damaged proteins is facilitated by the proteasome system, of which HSP70 and ubiquitin play crucial roles. For example, if repeated attempts at refolding have failed, HSP70 and co-chaperones (e.g. HSP40) present the damaged proteins to the proteasome system for degradation (Goldberg, 2003). Many of these proteins are subsequently marked with ubiquitin and are thereby targeted for degradation by the 26S proteasome (Glickman and Ciechanover, 2001). The observed increases in ubiquitin conjugates (Thompson and Scordilis, 1994; Stupka *et al.*, 2001), ubiquitin (Thompson and Scordilis, 1994; Willoughby *et al.*, 2000) and HSP70 (see section 2.4) levels of human muscle following a variety of exercise protocols are therefore thought to facilitate the targeting and removal of damaged and denatured proteins (Reid, 2005).

2.6.2.4 Muscle hypertrophy / fibre transition

Muscle fibre hypertrophy (an increase in cell size) functions to increase the force generating capacity of the muscle and is a particularly important adaptation after sprint or resistance type training (Maughan *et al.*, 1997). Given the enhanced myofibrillar protein synthesis that occurs with such training and the chaperone properties of HSPs, it is feasible that exercise-induced increases in HSPs also play an active role in the process of hypertrophy. Increased levels of HSP70 have indeed been observed in hypertrophying animal (Kilgore *et al.*, 1994; Locke *et al.*, 1994; O'Neill *et al.*, 2006) and human (Liu *et al.*, 2003; Brkic *et al.*, 2004) skeletal muscle compared with non-hypertrophied control skeletal muscle.

It is difficult to ascertain the role of the exercise-induced increase of HSPs in facilitating hypertrophy, however, as much of the above data is based on laboratory

models of hypertrophy. In these situations, compensatory overload of plantaris muscle (via surgical removal of the gastronemius and soleus) results in significant hypertrophy and a change in predominant phenotype towards slower fibre types that is also accompanied by increased HSP70 levels (Kilgore *et al.*, 1994; Locke *et al.*, 1994; O'Neill *et al.*,2006). O'Neill *et al.* (2006) recently demonstrated that hypertrophy of overloaded plantaris muscle still occurred despite blocking changes in fibre phenotype and the associated increases in HSP70 content, via thyroid hormone treatment. These data therefore suggest that significant hypertrophy can occur in the absence of increased HSP70.

Given that slower muscle phenotypes therefore appear critical for constitutive HSP70 expression, it is possible that much of the reported increases in HSP70 content of hypertrophied muscle simply reflect a general switch from fast to slow phenotypes. Indeed, Locke *et al.* (1994) demonstrated that when soleus muscle underwent fibre type profile shifts from type I to type II fibres (induced by treatment of 3.5.3'-triiodo-DL-thyronine), a decrease in HSP72 levels were observed. Future work should therefore examine muscle HSP levels in naturally occurring models of hypertrophy, particularly those of fast twitch fibres as such fibres are particularly sensitive to hypertrophy during resistance training (Maughan *et al.*, 1997). Nevertheless, the above data appear to demonstrate an essential role of HSP70 in muscle fibre transition from fast to slow type fibres, possibly to facilitate the increased rate of protein turnover inherent of these fibres (Obinata *et al.*, 1981).

2.6.2.5 Muscle atrophy

There are also reports that HSPs may play a role in the process of muscle atrophy (Ku *et al.*, 1995; Naito *et al.*, 2000; Selsby and Dodd, 2005). The initial loss of muscle protein during atrophy is primarily due to a decrease in the rate of protein synthesis whereas subsequent atrophy occurs by an increased rate of protein degradation (Thomason *et al.*, 1990). Naito *et al.* (2000) demonstrated that a prior period of whole body hyperthermia 24 h prior to 8 days of hindlimb unweighting significantly reduced the rate of disuse muscle atrophy in the soleus muscle of rodents. These authors suggested that the heat-induced expression of HSP72 in the soleus muscle

may have maintained protein synthesis by sustaining polypeptide elongation rate. Alternatively, the increased content of HSP72 following heat stress may have reduced the rate of proteolysis during unweighting by repairing the oxidative injury to muscle proteins that is now well documented during periods of disuse (Kondo and Itokawa, 1994; Lawler *et al.*, 2003; Powers *et al.*, 2005; Lawler *et al.*, 2006). Indeed, the addition of heat stress during an 8 day period of limb immobilization (which up-regulated HSP25 and HSP70) significantly attenuated the oxidative stress and atrophy of rodent soleus muscle, an effect attributed to the increased HSP mediated protection against oxidative injury (Selsby and Dodd, 2005).

Muscle atrophy is a well-documented feature of the ageing process (i.e. sarcopenia) and has been suggested to be due to increased oxidative stress (Powers *et al.*, 2005) and an accelerated rate of apoptosis in aged muscle (Dirks and Leeuwenburg, 2005). Ageing is associated with an attenuated ability to express HSPs following stress (Vasilaki *et al.*, 2002) and this lack of an ability to mount a stress response is thought to play a major role in the age related functional deficit that occurs in the skeletal muscle of elderly humans (Vasilaki *et al.*, 2002). Indeed, A.McArdle *et al.* (2004) demonstrated that lifelong overexpression of HSP70 in the skeletal muscle of transgenic mice provided protection against the fall in specific force associated with ageing. Overexpression of HSP70 also facilitated rapid and successful regeneration of muscles following contraction-induced damage.

The above data therefore suggest that therapy designed to maintain the stress response during normal ageing could impact considerably on the quality of life of the elderly. In this regard, it is tempting to speculate that life long physical activity may prove useful as an appropriate therapeutic intervention. Recent data has demonstrated that the maintenance of physical activity during ageing can preserve the ability of leukocytes from aged individuals to produce HSP72 following an exercise stress (Simar *et al.*, 2004). It remains to be demonstrated, however, whether life long exercise can maintain the ability of skeletal muscle to produce a stress response. Naito *et al.* (2001) demonstrated that 10 weeks of exercise training in aged rodents induces HSP72 synthesis in highly oxidative skeletal muscles although this response is blunted in fast glycolytic muscles. This lack of an HSP response in fast twitch

muscles may therefore, in part, explain the apparent predominance of atrophy of type II fibres during the ageing process (Young *et al.*, 1984, 1985).

2.6.2.6 Mitochondrial biogenesis

Endurance training or chronic stimulation of skeletal muscle stimulates mitochondrial biogenesis, increasing the overall number and volume of the organelle (Hood *et al.*, 2001). Mitochondria are never formed from *de novo* synthesis and the number and size of the organelle increases through the growth and division of existing mitochondria. Crucial to this process, is the increased transcription and translation of nuclear DNA encoded proteins and their subsequent import into the mitochondria. Efficient transport and import of such proteins requires a well-developed protein transport system of which cytoplasmic HSP70, mitochondrial HSP70 (mtHSP70 or GRP75) and HSP60 plays an integral role (Hood *et al.*, 2000). Precursor proteins made in the cytoplasm are chaperoned to the mitochondrial import machinery by HSP70. The matrix chaperone mtHSP70 'pulls' in the precursor across the inner membrane in an ATP dependent fashion (Koehler, 2000). Following the cleaving of the mitochondria-targeting signal sequences, the mature protein is refolded into its 3-dimensional structure by HSP60 and chaperonin 10 (Manning-Krieg *et al.*, 1991).

Given the essential roles of the above HSPs in facilitating biogenesis of mitochondria, it follows that the increased production of HSPs during repeated bouts of exercise (i.e. exercise training) may mediate exercise-induced mitochondrial biogenesis. Indeed, low frequency chronic stimulation of skeletal muscle ranging from 5 to 14 days elicits an increased protein content of HSP60 and mtHSP70 (Ornatsky *et al.*, 1995; Takahashi *et al.*, 1998; Gordon *et al.*, 2001), which was accompanied by accelerated rates of precursor import into the mitochondrial matrix (Gordon *et al.*, 2001). The more physiological stress of treadmill training has also been shown to increase HSP60 and mtHSP70 content of rodent muscle (Mattson *et al.*, 2000). The influence of training status on HSP60 content of human skeletal muscle, however, remains unclear.

2.6.3 Regulators of apoptosis

The cytoprotective properties of HSPs is largely considered attributable to their chaperone function where they operate by binding to misfolded and unfolded proteins and facilitate their refolding when cellular conditions become more favourable (Broome, 2003). However, HSPs are potent regulators of apoptosis (Takayama *et al.*, 2003) and thus it is feasible that HSPs may also exert their protective influences by promoting 'survival' pathways whilst simultaneously down-regulating 'death' pathways (Beere, 2005).

The increased expression of HSPs following acute and chronic exercise may therefore also play an important role as regulators of exercise-induced apoptosis via their interaction with intracellular signalling cascades, particularly the mitogen activated protein kinase (MAPK) family. The MAPK signalling cascade is a ubiquitously expressed intracellular network of phosphorylation cascades that link cellular changes in stress to changes in transcriptional activity (Chau Long *et al.*, 2004). Several members of the MAPK family, including the c-Jun NH_2 terminal kinase (JNK) and p38 stress kinase, have been shown to be phosphorylated in human skeletal muscle following cycling exercise (Widegren *et al.*, 1998), marathon running (Boppart *et al.*, 2000) and resistance exercise (Thompson *et al.*, 2003). Damaging or unaccustomed exercise may lead to exercise-induced apoptosis whereby the activation of JNK and the associated leakage of cytochrome C from the mitochondria into the cytosol is believed to be one of the key precipitating steps (Phaneuf and Leeuwenburgh, 2001). Several members of the HSP family, particularly HSP27 and HSP70, can modulate cell signalling at several sites and thus it can be postulated that HSPs may also exert their protective influence by suppression of the apoptotic pathway in addition to their more established role as molecular chaperones (Gabai and Sherman, 2002). It has therefore been suggested that whether the muscle cell undergoes repair or apoptosis following exercise may ultimately depend on the availability of HSPs to stabilize and chaperone denatured proteins while simultaneously suppressing key stages of the apoptotic pathway (Noble, 2002).

Considering the ability of exercise training to up-regulate HSP expression in both cardiac and skeletal muscle, it is possible that training-induced increases in HSP content play an important role in down-regulating apoptosis. This is of particular interest for aged muscle where an increased rate of apoptosis is thought to be a possible contributor of sarcopenia (Dirks and Leeuwenburgh, 2005). The effects of exercise training on muscle apoptosis are now beginning to be investigated. Recent data demonstrated that periods of aerobic treadmill training in rats increased the Bcl-2/Bax ratio of soleus muscle whilst increasing HSP70 expression, thereby promoting a clear decrease in the rate of apoptosis occurring from the mitochondrial pathway (Siu *et al.*, 2004). Exercise training can also induce protective effects against elevated apoptosis in the aged rodent heart (Kwak *et al.*, 2006). Together, these data suggest that life long physical activity may reduce the loss of skeletal and cardiac myocytes via an up-regulation of HSPs and a simultaneous down-regulation of apoptotic proteins.

2.6.4 Summary

HSPs are now thought to perform a variety of physiological, pathophysiological and functional roles within the cell. In relation to exercise, the increased production of HSPs likely function to facilitate cellular repair, promote remodelling and to provide cytoprotection against further insults. With the advancement of cellular and molecular techniques, it is likely that the list of known functions of HSPs will continue to grow. The precise biological roles that these proteins play in relation to exercise will also likely be soon understood.

2.7 SUMMARY AND CONCLUSIONS

From their original role as molecular chaperones, it is apparent that the study of HSPs has significantly advanced in recent years. The addition of exercise to the list of known inducers of HSPs has opened a new field of investigation. In this regard, the exercise-induced accumulation of HSPs likely functions to restore cellular homeostasis, provide cytoprotection against related and non-related stressors and also facilitate important cellular remodelling processes. However, despite over a decade of research, there are many fundamental questions that remain unanswered. This is particularly the case in relation to human tissue where data is minimal and inconclusive. For example, the response of the major HSP families remains to be simultaneously examined following an acute exercise stress. As such, it is possible that specific HSPs may be differentially up-regulated according to particular characteristics of the exercise protocol. The precise stressor or stressors that is responsible for inducing an increased production of HSPs following exercise also remains to be defined. Furthermore, the relative contribution of possible signals or stresses may also vary according to the nature of the exercise stress. The influence of exercise training on basal muscle HSP levels and on the magnitude of the exercise-induced production of HSPs also remains undefined. Clearly, the area of HSPs, muscle and exercise is at an exciting stage and it is hoped that these questions will be answered in the experimental studies undertaken in the present thesis.

Chapter 3

General Methodology

3.1 General Methodology

Many of the methods undertaken in this thesis are replicated throughout Studies 1-5. For brevity, these methods have therefore been detailed in the following Chapter with reference to this section made in each of the experimental Chapters, where appropriate.

3.1.1 Location of Testing and Ethical Approval.

All of the experimental protocols and related procedures undertaken were conducted in the Exercise Physiology Laboratories of the Research Institute for Sport and Exercise Sciences. All of the biochemical procedures were performed in the School of Clinical Sciences at the University of Liverpool. All of the experimental protocols and related procedures were approved by the Ethical Committee of Liverpool John Moores University.

3.1.2 Subjects.

All of the subjects who volunteered to participate in each study were young healthy untrained, recreationally active or aerobically trained males. Subjects were classified as untrained, active or trained according to their $\dot{V}O_{2max}$ (see section 3.3), lactate threshold (see section 3.4) and number of hours involved in physical activity per week (American College of Sports Medicine, 1998). A comparison of subjects' physical and physiological characteristics is shown in Table 3.1. There was a significant difference ($P<0.05$) between groups in $\dot{V}O_{2max}$, lactate threshold (both % of $\dot{V}O_{2max}$ and running speed at lactate threshold) and number of hours involved in physical activity, which was present between all pair-wise comparisons.

All subjects gave written informed consent to participate after details and procedures of the study had been fully explained. Subjects refrained from exercise throughout the course of each study and from alcohol and caffeine intake for at least 24 h prior to

any of the testing or muscle biopsy sessions. All subjects were non-smokers, had no history of neurological disease or musculoskeletal abnormality and none were under any special pharmacological treatment during the course of the study. Participation was entirely voluntary and subjects were free to withdraw from the study at any time.

Table 3.1 – Physical and physiological characteristics of the untrained, active and trained subjects who participated in the experimental studies (mean ± SD).

	UNTRAINED	ACTIVE	TRAINED
Age (years)	29 ± 6	24 ± 3	27 ± 6
Height (m)	1.76 ± 0.05	1.79 ± 0.05	1.76 ± 0.04
Weight (kg)	78.1 ± 15.5	77.8.1 ± 7.3	75.1 ± 6.8
$\dot{V}O_{2max}$ ($ml.kg^{-1}.min^{-1}$)	48.9 ± 2.9 *	56.4 ± 3.9 *	65.3 ± 4.9 *
LT (% of $\dot{V}O_{2max}$)	64.5 ± 1.9 *	70.5 ± 2.7 *	76.3 ± 2.3 *
Running speed @ LT ($km.h^{-1}$)	9.4 ± 1 *	11.5 ± 0.6 *	13.8 ± 0.4 *
Hours of activity per week	0.7 ± 0.8 *	2.4 ± 0.7 *	7.8 ± 1.1 *

* denotes significant difference between all pair-wise comparisons, $P<0.05$

3.1.3 Anthropometry

Subjects' height was measured whilst standing in the Frankfurt plane using a stadiometer (Seca, Birmingham, U.K). Subjects' body mass was recorded, whilst nude, using precision calibrated weighing scales (Seca, Birmingham, U.K).

3.1.4 Exercise and Heating Protocols

Each exercise related study involved treadmill running as the exercise protocol. All tests were conducted on a motorised treadmill (Woodway, Auf-Schrauben, Germany; see Figure 3.1). Study 4 (Chapter 6) involved a 1 h passive heating stress as described in section 6.2.

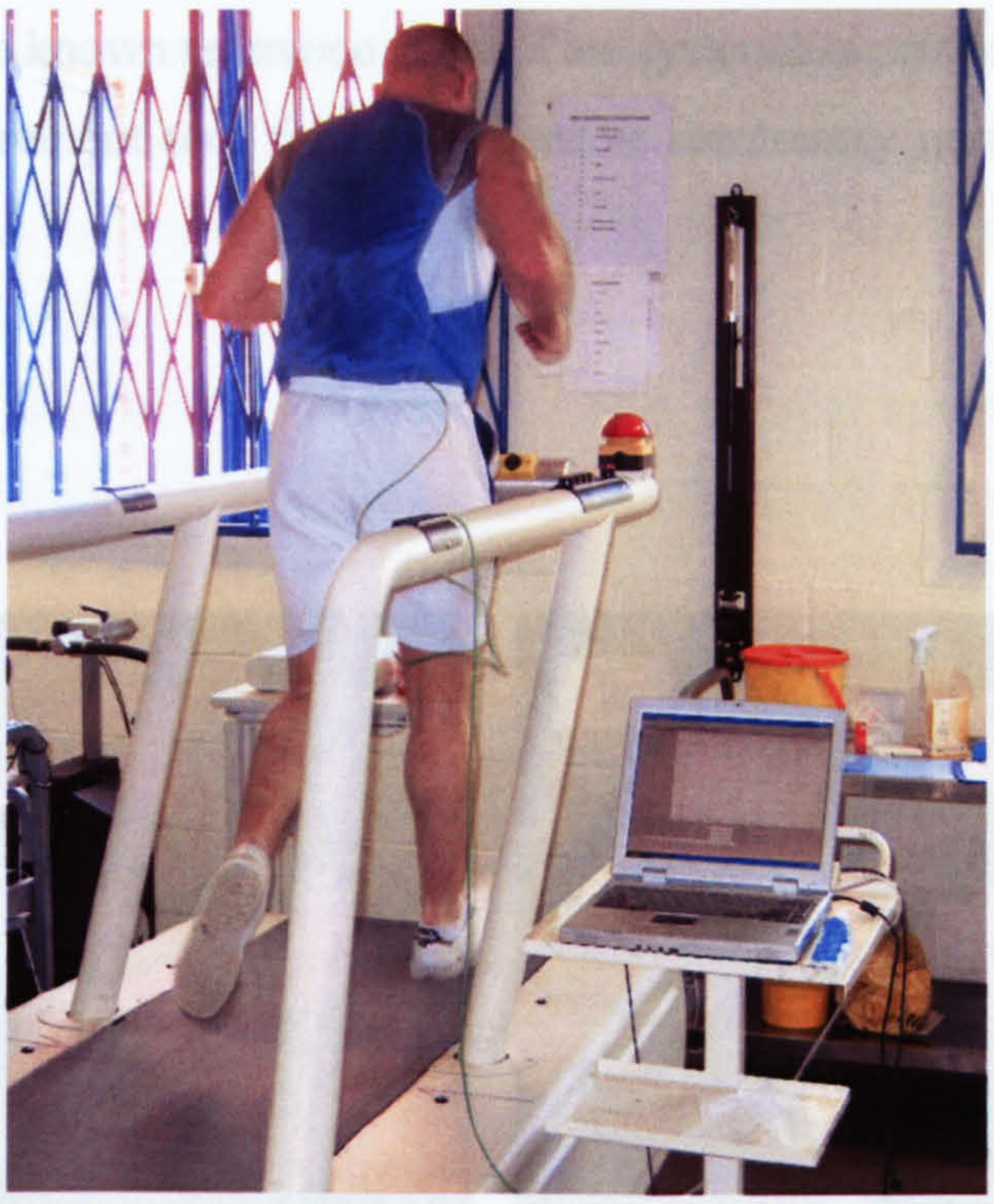

Figure 3.1 – Illustration of subject performing running exercise protocol on the motorised treadmill that was used in all exercise-related studies.

3.2 Cardio-Respiratory Measurements

3.2.1 Heart rate

Subjects were fitted with a short–range radio telemetry system for the measurement of heart rate (Polar 610i, Kempele, Finland) in all exercise related and heating experiments.

3.2.2 Assessment of respiratory gases during exercise

Subjects were fitted with a facemask (for assessment of $\dot{V}O_{2max}$ and lactate threshold) or mouthpiece (for durations of sampling of 5 mins) for measurement of respiratory gases during exercise (see Figure 3.2). Expired fractions of oxygen and carbon dioxide were averaged over each 10-second period and were analysed via an on-line gas analysis system (MetaLyzer 3B, Cortex Biophysic GmbH, Leipzig, Germany) after calibration with known reference gases. This system has previously been deemed as a reliable measurement tool for assessment of respiratory gases during exercise (Meyer *et al.*, 2001).

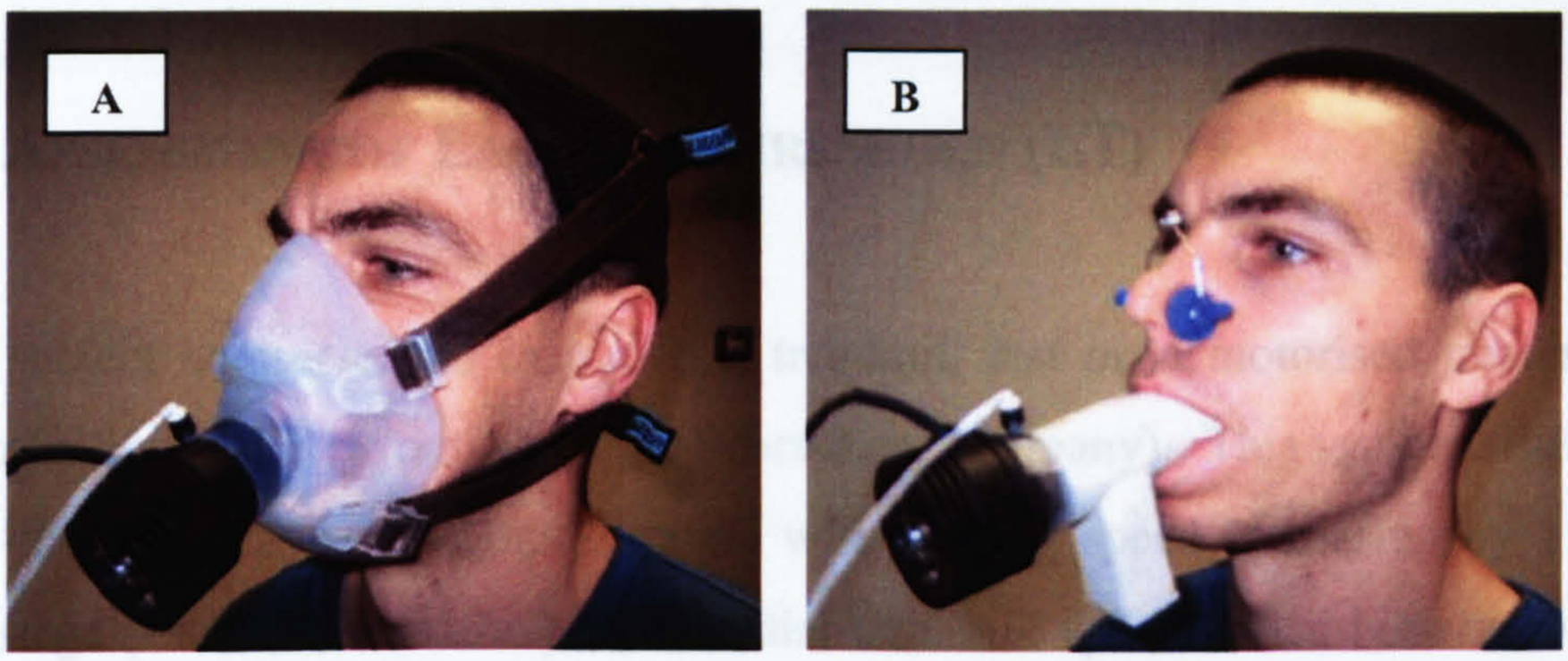

Figure 3.2 – Subjects were fitted with (A) a facemask or (B) a mouthpiece during exercise.

3.3 Assessment of Maximal Oxygen Uptake ($\dot{V}O_{2max}$)

Each subject completed a standard incremental treadmill test on a motorised treadmill (Woodway, Auf-Schrauben, Germany) for determination of $\dot{V}O_{2max}$. For active and trained subjects, the initial treadmill speed was set at 10 km.h^{-1} and increased by 2 km.h^{-1} every 2 min thereafter. For untrained subjects, the initial treadmill speed was set at 6 km.h^{-1} and also increased by 2 km.h^{-1} every 2 min thereafter. After completion of the 2 min stage at 16 km.h^{-1}, the treadmill was inclined by 2% every 2 min until test termination. Termination of the test occurred upon volitional exhaustion despite strong verbal encouragement. The $\dot{V}O_{2max}$ was taken as the highest $\dot{V}O_2$ value attained in any 10 s period and was stated as being achieved by all of the following end-point criteria: 1) heart rate within 10 b.min^{-1} of age-predicted maximum, 2) RER > 1.1, 3) plateau of oxygen consumption despite increasing workload and 4) blood lactate concentration > 8 mmol.l^{-1} (Bird and Davison, 1997). Expired fractions of oxygen and carbon dioxide were averaged over each 10-second period and were analysed via an on-line gas analysis system (MetaLyzer 3B, Cortex Biophysic GmbH, Leipzig, Germany) after calibration with known reference gases. Heart rate (HR) was measured continuously using short-range radio telemetry (Polar 610i,Kempele, Finland). Fingertip capillary blood samples were collected immediately post-exercise and analysed for whole blood lactate concentration, in duplicate, using an automatic portable analyser (Lactate Pro, Arkray, Kyoto, Japan).

3.4 Assessment of Lactate Threshold (LT)

Each subject completed an incremental treadmill test on a motorised treadmill for determination of LT (Woodway, Auf-Schrauben, Germany). This test consisted of 5-7 sub-maximal stages of 4 min duration with running speed increasing by 1 km.h^{-1} each stage (Carter *et al.*, 1999). The initial treadmill speed varied between subjects (6-10 km.h^{-1}) according to each subject's $\dot{V}O_{2max}$ (Carter *et al.*, 1999). Fingertip capillary blood samples were collected immediately prior to beginning the test and during 30 s rest periods between each increment (Gullstrand *et al.*, 1994). Whole blood lactate concentration was determined immediately, in duplicate, using an

automatic portable analyser (Lactate Pro, Arkray, Kyoto, Japan). The analyser was calibrated with a 5 mmol.l^{-1} lactate standard before each individual test and after every 6 samples. Oxygen uptake was measured continuously throughout the test (MetaLyzer 3B, Cortex Biophysic GmbH, Leipzig, Germany) with the $\dot{V}O_2$ during the last minute of each stage being retained for analysis (Carter *et al.*, 1999). Plots of blood lactate concentration against running speed and $\dot{V}O_2$ were subsequently distributed to two independent and experienced reviewers who determined the lactate threshold as the first sustained increase in blood lactate above baseline (Carter *et al.*, 1999). The reliability of data plot assessment was high both between reviewers (r = 0.98) and within reviewers (r = 0.99).

3.5 Assessment of Delayed Onset Muscle Soreness (DOMS)

3.5.1 Pain Diagrams

Subjects completed a pain diagram (Close *et al.*, 2004) pre- and post-exercise which involved rating their perceived muscle soreness of the gastrocnemious, tibialis anterior, hamstrings, quadriceps, gluteals, (both left and right sides) and lower back muscles. Subjects rated the extent of soreness according to a modified Borg Scale with 0 = no pain and 10 = unbearable pain. Scores for the 11 sites were then accumulated and divided by the number of sites assessed which was then reported as an overall indicator of whole body soreness (Close *et al.*, 2004). An illustration of the pain diagram is shown in Figure 3.3.

3.5.2 Visual Analogue Scale (VAS)

Subjects also evaluated the extent of muscle soreness, specific to the quadriceps, using a 100 mm visual analogue scale in which 0 = no pain and 100 = unbearable (Close *et al.*, 2004). Subjects were asked to place a mark on the line that corresponds with the amount of pain felt in the quadriceps. The distance from 'no pain' was measured and this distance was used to quantify the amount of soreness. An illustration of the VAS is shown in Figure 3.4.

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Figure 3.3 – Pain diagram used to assess whole body soreness (Close *et al.*, 2004).

Figure 3.4 – Visual Analogue Scale to assess soreness specific to the quadriceps (Close *et al.*, 2004).

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3.6 MEASUREMENT OF PSYCHO-PHYSIOLOGICAL VARIABLES

3.6.1 Ratings of Perceived Exertion (RPE)

Subjects reported ratings of perceived exertion during exercise according to a 15-point Borg Scale (Borg, 1970). The category ratio scale that was used is displayed in Table 3.2.

3.6.2 Ratings of Thermal Comfort (RTC)

Subjects reported ratings of thermal comfort during exercise or heating according to a 9-point thermal comfort rating scale (Toner *et al.*, 1986). The category ratio scale that was used is displayed in Table 3.3.

Table 3.3. – Thermal comfort scale used for subjects' ratings of their perceived thermal comfort during exercise or heating (Toner *et al.*, 1986).

Rating	Description
1	Very Cold
2	Cold
3	Cool
4	Slightly Cool
5	Neutral
6	Slightly Warm
7	Warm
8	Hot
9	Very Hot

3.7 THERMOREGULATORY VARIABLES

3.7.1 Core Temperature

Core temperature was measured rectally using a rectal probe placed 10 cm beyond the anal sphincter (ELLAB, Denmark). Core temperature was monitored continuously during exercise or heating (CTF9004, ELLAB, Denmark) with the core temperature at 5 min intervals being recorded and retained for analysis.

3.7.2 Muscle Temperature

Muscle temperature of the vastus lateralis was measured immediately pre- and post-exercise using a needle thermistor (CTF9004, ELLAB, Denmark) inserted to a depth of 3 cm (Saltin *et al.*, 1968). During the heating protocol, muscle temperature was measured immediately pre- and at 20 min intervals during the 1 h protocol. When the needle thermistor was removed, the injection site was cleaned with a sterile alcohol injection swab and covered with waterproof dressing.

3.8 NON-DAMAGING RUNNING EXERCISE PROTOCOL

In each exercise related study examining the HSP response to exercise, untrained, active or trained subjects performed 45 min of running exercise on a motorised treadmill (Woodway, Auf-Schrauben, Germany; see Figure 3.1) at a treadmill speed corresponding to the subjects' lactate threshold (see section 3.4). This intensity of exercise (as opposed to % of $\dot{V}O_{2max}$) was chosen so as to normalise the exercise intensity between individuals with different aerobic capacities (Baldwin *et al.*, 2000). This exercise intensity also compares well with similar intensities previously used in treadmill protocols examining the heat shock response to exercise (Puntschart *et al.*, 1996; Walsh *et al.*, 2001). This protocol was deemed non-damaging for active and trained subjects in Study 2 and for untrained subjects in Study 5, where non-damaging is defined as exercise that induces no overt structural or functional damage to the muscle (Vasilaki *et al.*, 2006).

Ratings of perceived exertion (see section 3.6.1), thermal comfort (see section 3.6.2) and heart rate (see section 3.2.1) were recorded at 5 min intervals throughout exercise. Oxygen uptake was also measured (see section 3.2.2; Figure 3.2B) for 5 min periods between 5-10 mins, 20-25 mins and 35-40 mins of exercise. Fingertip capillary blood samples were obtained at 15 min, 30 min and 45 min and analysed immediately, in duplicate, for whole blood lactate concentration (Lactate Pro, Arkray, Kyoto, Japan) to verify the intensity of exercise. Core temperature was measured continuously during exercise (see section 3.7.1) and muscle temperature of the vastus lateralis was measured immediately pre- and post-exercise (see section 3.7.2). The ambient temperature of the laboratory during each exercise session was approximately 19 ± 0.8°C. Fluid intake was not permitted at any time during exercise.

3.9 Procurement and Storage of Blood Samples

Venous blood samples (6 ml) were drawn from a superficial vein in the anti-cubital crease of the forearm using standard veinepuncture techniques (Vacutainer Systems, Becton, Dickinson, Europe; Figure 3.5) and immediately dispensed into a pre-coated serum separation tube (HMS, Northampton, U.K). The sample was left at room temperature for ~30 mins prior to being centrifuged at 15 000 rpm at 4° C for 15 min (Mistral 30001, MSE, Cambridge, U.K). The serum was then removed and stored at –70°C for later determination of creatine kinase activity according to a modification of the spectrophotometric method of Jones *et al.* (1983) (see section 3.12.1).

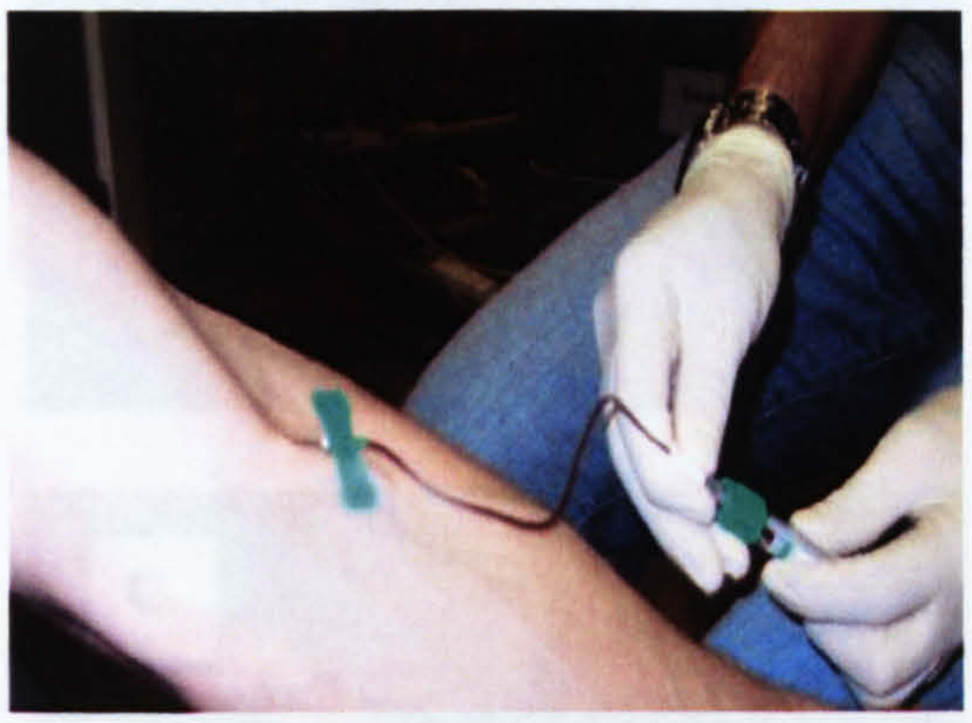
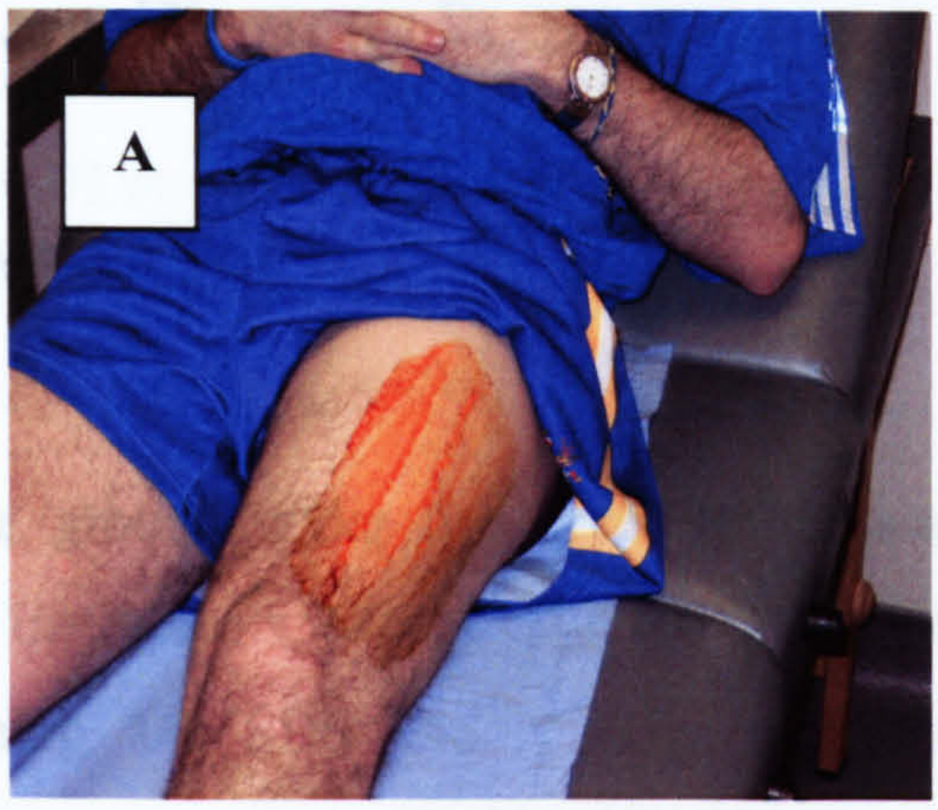
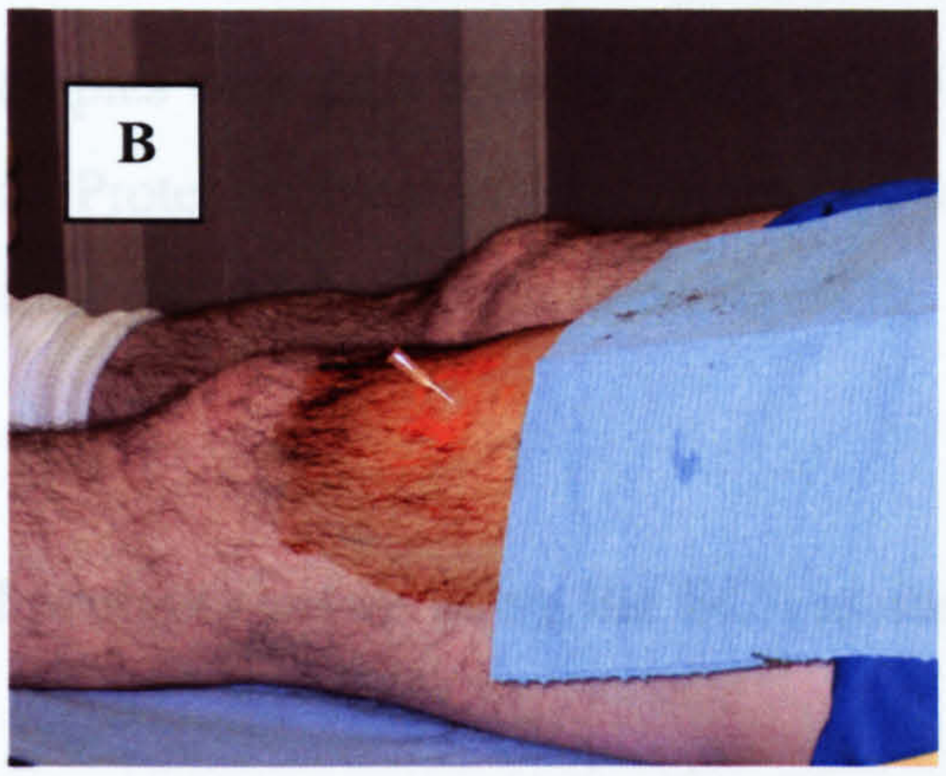


Figure 3.5 – Illustration of the vacutainer blood collection system used to obtain venous blood.

3.10 Muscle Biopsies

Muscle biopsies were taken from the vastus lateralis under local anesthesia (0.5% marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL, USA). Samples obtained (~50mgs) were immediately frozen in liquid nitrogen and stored at –70°C for later analysis. An illustration of the Pro-Mag biopsy gun is shown in Figure 3.6. Previous data have shown that the process of serial muscle biopsies *per se* is insufficient to induce increases in muscle HSP content (Khassaf *et al.*, 2001).





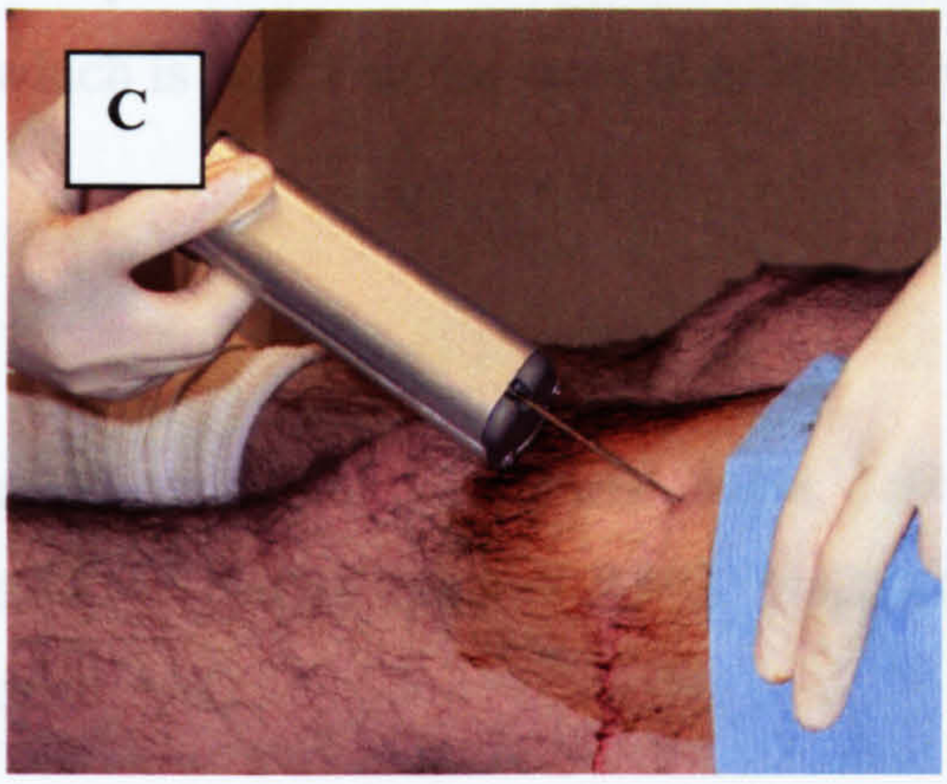


Figure 3.6 – Illustration of the muscle biopsy procedure. (A) The vastus lateralis was firstly shaved and cleaned with Betadine solution and (B) subsequently injected with local anaesthetic. (C) Approximately 50 mg of muscle was then removed using the Pro-Mag 2.2 Biopsy Gun.

3.11 Analysis of HSP content of skeletal muscle by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

3.11.1 Sample preparation

Muscle samples were ground under liquid nitrogen. Samples were subsequently homogenised (TRI-R Instruments, Model K43, Rockville Centre, NY) on ice in 120 µl of buffer containing 1% SDS and a range of protease inhibitors (1 mM iodoacetamide, 1mM benzethonium chloride, 5.7 mM phenylmethylsulfonyl fluoride and 5 mM EGTA). Samples were then centrifuged at 10 000 *g* for 10 mins at 4 °C to clarify the supernatant. Protein content of the supernatant was determined using the bicinchoninic acid method (BCA) for protein determination.

3.11.2 Assessment of protein content using the BCA method

Protein content of the supernatant was measured using the BCA protein assay kit (Sigma, Dorset, UK) which is based on the method of Smith *et al.* (1985).

Reagents:

Reagent A: BCA solution containing 25 mM BCA-Na, 160 mM $NaHCO_3$, 7.0 mM Na_2 tartrate, 0.1 mM NaOH and 0.95% $NaHCO_3$, pH 11.25 (Sigma Immunochemicals, Dorset, UK).

Reagent B: 160 mM $CuSO5H_2O$ (Sigma Immunochemicals, Dorset, UK).

Protocol:

A range of standards between 0-250 µg/ml was prepared from a stock solution of 1 mg/ml Bovine serum albumin (BSA) in 0.15 M NaCl with 7.7 mM NaN_3. Reagent C was prepared immediately before use by the addition of 500 µl of Reagent B to 25 ml

of Reagent A. Twenty µl of the standard, blank or sample and 200 µl of Reagent C were placed in a 96 microtitre plate, mixed and incubated at 50°C for 30 mins. Samples were then cooled at room temperature and the absorbance of standards and samples measured at 570 nm using a microplate reader (Benchmark, Biorad, UK). The protein content of each sample was then calculated from the standard curve. All measurements were repeated in duplicate.

3.11.3 Preparation of polyacrylamide gradient gels

Reagents:

Stock acrylamide solution: 30% acrylamide, 0.8% bisacrylamide cross-link in dH_2O (Protogel, National Diagnostics, USA).

Gel Buffer: 1.5M Tris/HCL, 0.384% SDS, pH 8.8 (Protogel Buffer, National Diagnostics, USA).

Stacking Buffer: 0.5M Tris/HCL. 0.4% SDS, pH 6.8 (Protogel Stacking Buffer, National Diagnostics, USA).

12 % Acrylamide solution (100 ml):

40 ml stock acrylamide solution

26 ml gel buffer

34 ml dH_2O

4% stacking gel solution (100 ml):

13 ml stock acrylamide solution

25 ml stacking buffer

62 ml dH_2O

Protocol:

Twelve % solution of acrylamide with 0.8% bisacrylamide cross-link was prepared as described as above. Gel formation was catalysed by the addition of 100 µl of 10%

aqueous ammonium persulphate solution (APS) and 10 µl of NNN'N'-tetramethylethylene-diamine (TEMED) to 10 ml of the 12% acrylamide solution. The gel solution was immediately poured between gel plates (8 x 10 cm) with 2 mm spacers and allowed to set for 10 to 20 mins. A 4% stacking gel solution was made as described as above. Fifty µl of 10% aqueous APS and 10 µl of TEMED were added to 10 ml of the 4% gel solution . The 4% stacking gel solution was poured on top of the of the 12% gel to form a 2 – 2.5 cm stacking gel and a well comb placed in position to facilitate sample loading.

3.11.4 Preparation of samples for SDS-PAGE and Western Blotting

Reagents:

Laemmli Buffer: 46.03 mg/ml SDS, 20.9% glycerol, 2.1% (v/v) B-mercaptoethanol, 0.052 mg/ml bromophenol blue in 0.128M Tris/HCL buffer, pH 6.8.

Protocol:

Fifty µg of total cellular protein (as determined from section 3.9.2) was boiled for 5 mins in a water bath in an equal volume of Laemmli buffer. The cooled sample was then applied to the polyacrylamide gel solution.

3.11.5 Electrophoresis of proteins

Reagents:

Electrophoresis buffer: 10 X Tris/ Glycine/ SDS (0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS; National Diagnostics, Hessle, Hull, UK).

Protocol:

Electrophoresis was carried out using an Anachem Electrophoresis tank with an LKB Power Pack cooled with H_2O at a constant current of approximately 20 mA per gel until the samples had run through the 4% stacking gel. The current was then

increased to 40 mA until the visible bromophenol dye was approximately 1 cm above the bottom of the gel (approximately 1.5 – 2 h). The separated proteins were then transferred from the gel onto a nitrocellulose membrane by western blotting. All gels were run in duplicate.

3.11.6 Western blotting of separated proteins

Reagents:

Anode 1 Buffer: 0.3M Tris in a 20% methanol solution, pH 10.4

Anode 2 buffer: 25 mM Tris in a 20% methanol solution, pH 10.4

Cathode buffer: 40 mM 6-amino n hexanoic acid in a 20% methanol solution, pH 7.6.

Protocol:

The Multiphor II discontinuous blotting system (Pharmacia, Milton Keynes, UK) consists of two graphite plate electrodes with Whatman No 1 filter paper used as a buffer reservoir. Following electrophoresis, the gel was removed from the glass plates and placed on top of nitrocellulose (0.45 μM pore size; Anderman & Co. Ltd, Surrey, UK) and this was sandwiched between the electrodes (as shown in Figure 3.7). A constant current density of 0.8 mA/cm was applied to the system for a minimum time of 1.5 h at room temperature. Post-blotting staining of gels has revealed that this was sufficient to produce maximal transfer of proteins to the nitrocellulose.

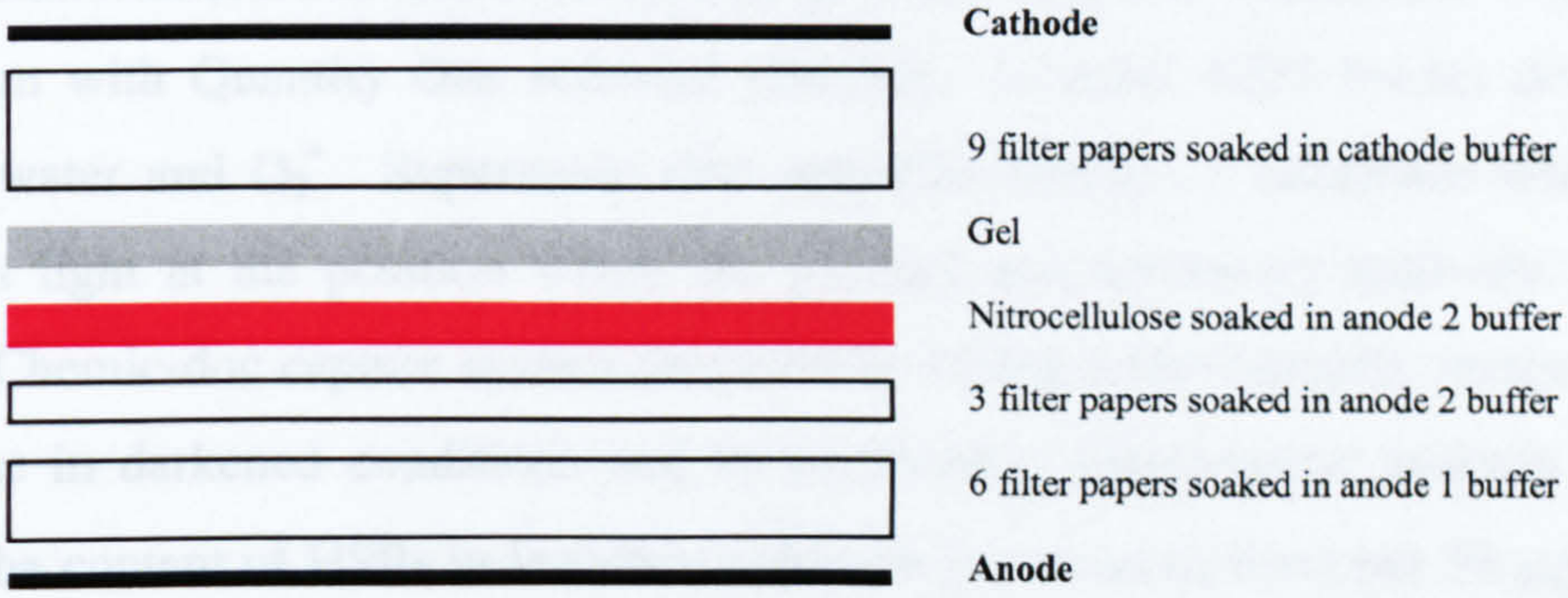


Figure 3.7 – Schematic illustration of western blotting procedure.

3.11.7 Processing and development of the nitrocellulose membrane

Reagents:

PBS solution: 0.05M KH_2PO_4, 0.05M NA_2HPO_4, 1.3M NaCl in dH_2O, pH 7.2.

PBS solution: 0.05M KH_2PO_4, 0.05M NA_2HPO_4, 1.3M NaCl in dH_2O, pH 6.0.

PBS Tween: 0.05% (v/v) polyoxyethylene-sorbitan monolaurete (Tween 20) in PBS solution, pH 7.2.

Protocol:

Following electroblotting, the nitrocellulose was removed and placed in 100 ml of a blocking solution of 5 g powdered milk/100 ml PBS Tween for either 1 h at room temperature or overnight at 4 °C. The membrane was then washed for 3 X 10 mins in PBS Tween. The nitrocellulose membrane was then analysed for various HSPs and antioxidant proteins. The nitrocellulose was agitated for 1 h in 10 ml of a solution of PBS Tween containing the primary antibody (at the concentration listed in Table 3.4) for 1 h at room temperature. The membrane was then washed in PBS Tween for 3 X 10 mins and placed into the appropriate peroxidise (horseradish peroxidise, HRP) labelled secondary antibody solution in PBS Tween containing 25% FCS as a non-specific blocking agent for 1 h at room temperature. The membrane was then washed for 3 X 10 min in PBS Tween followed by 10 min in PBS pH 6.0 (the optimal pH for peroxidase activity). Bands were subsequently visualised on the membrane using an enhanced chemiluminescence detection system (Pierce, UK) and Chemi-doc image capture system with Quantity One software (Biorad). In brief, HRP breaks down peracid into water and $O_2^{\bullet}$. Superoxide then activates luminol, a substance which starts to emit light at the position where the primary and secondary antibody are bound. The Chemic-doc capture system functions by taking a photographic image of the membrane in darkened conditions and by performing densiometric analysis on each band. The content of HSPs in the vastus lateralis (i.e concentration per 50 μg of cellular protein) was expressed as a percentage of pre-intervention (exercise or heating) for each individual subject.

Table 3.4 – Source, species and dilution factors required for each protein antibody.

ANTIBODY	SOURCE	SPECIES	PRIMARY ANTIBODY DILUTION	SECONDARY ANTIBODY DILUTION
alpha B crystallin	Stressgen	Rabbit	1:1000	1:1000
HSP27	Stressgen	Rabbit	1:2500	1:1000
HSP60	Sigma	Mouse	1:500	1:1000
HSP70	Stressgen	Mouse	1:1000	1:1000
HSC70	Stressgen	Rat	1:1000	1:1000
MnSOD	Stressgen	Rabbit	1:2500	1:1000

3.11.8 Removing antibodies and re-probing the nitrocellulose membrane

Reagents:

100 mM B-mercaptoethanol (Sigma Immunochemicals, Dorset, UK)

2% (w/v) SDS

62.5 mM Tris/HCl, pH 6.7

PBS Tween, pH 7.2

Blocking solution containing 5g powdered milk/100 ml PBS Tween

Protocol:

Following investigation of a particular HSP, the antibodies can be removed and the nitrocellulose membrane can be re-analysed for other proteins. After exposure to ECL detection solution (Amersham, UK), the membrane was washed in PBS Tween for 10 mins and then incubated in a solution containing 100 mM B-mercaptoethanol, 2% SDS, 62.5 mM Tris/HCl, pH 6.7 for 30 mins at 50°C. The membrane was then washed for 2 X 10 mins in PBS Tween solution at room temperature. Finally, the membrane was placed in 100 ml of blocking solution for 1 h at room temperature or

alternatively at 4°C overnight. The membrane was then analysed for the content of other HSPs as described in section 3.11.7.

3.12 SPECTROPHOTOMETRIC ASSAYS FOR ANTIOXIDANT ENZYMES

3.12.1 Spectrophotometric assay for SOD activity based on the reduction of cycochrome c

This assay is based on the production of the superoxide radical by xanthine/xanthine oxidase. These radicals react with the oxidised form of cytochrome c to produce a reduced form of cyochrome c that absorbs light at 550 nm. Thus, xanthine/xanthine oxidase is used as a source of superoxide and cytochrome c is used as the indicating scavenger for the radical. SOD quenches this reaction; thus, the greater the activity of SOD the greater the quenching. This is assay is based on the method of Crapo *et al.* (1984).

Reagents:

All reagents were prepared in 50 mM potassium phosphate containing 0.1 mM EDTA, pH 7.8.

0.1 mM Ferricytochrome c

0.5 mM xanthine

Stock xanthine oxidase

50 mM potassium phosphate containing 0.1 mM EDTA, pH 7.8

Protocol:

Fifty µl of cytochrome c, 50 µl of xanthine and 400 µl of the phosphate buffer were placed in a cuvette. The reaction was initiated by the addition of 5 µl of stock xanthine oxidase diluted to give a rate of increased absorbance at 550 nm (at 25 °C) of 0.02 AU per minute. The reaction was monitored using a spectrophotomoter (CECIL CE594/ Double Beam Spectrophotometer, Cambridge, UK). Another reaction

mixture was then prepared in which 50 µl of the sample replaced an equal volume of the buffer and the rate was again recorded after dilution of xanthine oxidase. The ability of the sample to quench the production of the superoxide radical and so the reduction of cytochrome c was determined. All measurements were repeated in duplicate.

3.12.2 Spectrophotometric assay for catalase activity

The spectrophotometric assay of catalase (Claiborne, 1985) catalyses the decomposition of hydrogen peroxide to water and oxygen. The catalytic decompostion of hydrogen peroxide by catalase can be followed by ultraviolet spectroscopy due to the absorbance of hydrogen peroxide in this region. At 240 nm, the molar extinction coefficient for hydrogen peroxide at 43.6 $M^{-1}cm^{-1}$.

Reagents:

30% hydrogen peroxide

50 mM potassium phosphate buffer, pH 7.0

Protocol:

Since hydrogen peroxide degrades rapidly upon storage, the concentration of the stock hydrogen peroxide solution (normally 8.8 to 9.1 M) was measured by diluting hyfrogen peroxide in distilled water (1/800 dilution). The absorbance at 240 nm of the diluted peroxide sample was then recorded (CECIL CE594/ Double Beam Spectrophotometer, Cambridge, UK) against a matched quartz cuvette containing distilled water. The concentration of the stock hydrogen peroxide was calculated from the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$. A solution of 19 mM hydrogen peroxide was prepared in 50 mM potassium phosphate buffer, pH 7.0. Five hundred µl of this solution and 5 µl of sample were placed into a quartz cuvette and the decrease in absorbance (at 240 nm, 25 °C) was monitored. The specific activity of

catalse is defined in terms of μm of hydrogen peroxide consumed per minute per milligram of protein. All measurements were repeated in duplicate.

3.13 ANALYSIS OF BLOOD VARIABLES

3.13.1 Serum creatine kinase (CK) activity

Activity of CK was calculated from the conversion of $NADP^+$ to NADPH where one unit of CK activity is equivalent to the conversion of 1μmol of creatine phosphate substrate per minute at 20 °C (Jones *et al.*, 1983).

Reagents:

Cocktail Solution: 2 mM DL-Dithiothreitol (DTT)

10 mM Adenosine 5'-diphosphate (ADP)

20 mM Adenosine 5'-monophospahte (AMP)

40 mM phosphocreatine (PC)

2 mM B–Nicotinamide adenine dinucleotide phosphate (B-NADP)

5 mM glucose

40 mM TEA buffer, pH 7.2

5.4 U/l Hexokinase /glucose-6-phosphate dehydrogenase (HK/G6PDH)

10 mM NADPH:

Protocol:

A range of NADPH standards between 10 and 0.078 mM was prepared from a stock solution containing 16.6 mg of NADPH in 2 ml of TEA buffer (pH 7.2). Two hundred μl of the cocktail solution was added to 20 μl of the serum sample. The absorbance of samples was measured at 340 nm for 10 mins using a microplate reader

(Benchmark, Biorad, UK). Two hundred µl of standards was placed in a microplate reader and the absorbance measured at 340 nm using a microplate reader (Benchmark, Biorad, UK). TEA buffer was used as the blanks. The CK activity was calculated based on the production of NADPH and was expressed in $IU.L^{-1}$. All measurements were repeated in duplicate.

Chapter 4

Theoretical and Methodological Studies

The work presented in this Chapter consists of two studies that aimed to develop a non-damaging treadmill protocol to utilise as a stimulus to initiate the exercise-induced expression of HSPs (where non-damaging is defined as exercise that induces no overt structural or functional damage to the muscle). Study 1 (section 4.1) evaluated the reliability of maximal quadriceps isometric muscle force and voluntary activation across a timescale relating to how these variables were to be assessed in Study 2 (section 4.2). Aspects of this work have been published in the European Journal of Applied Physiology (see Appendix 1) and were also presented at The Physiological Society, King's College London Meeting, December 2004 (poster communication, see Appendix 3)

Study 2 (section 4.2) then used these variables along with circulating levels of creatine kinase and subjective feelings of muscle soreness to provide an indirect indicator of muscle damage in a trained and active male population following a 45 min running protocol at a speed corresponding to the lactate threshold. When the protocol was confirmed as non-damaging, 2 subjects re-performed the exercise bout 6-8 weeks later so as to provide pilot data ensuring that the exercise protocol was sufficient to initiate a heat shock response in skeletal muscle. This work was presented at the Liverpool Mini-Symposium on ROS, Muscle and Ageing, May 2005 (poster communication, see Appendix 4). Aspects of this work have also been published in the Journal of Applied Physiology (see Appendix 2).

4.1 THE RELIABILITY OF MAXIMAL QUADRICEPS ISOMETRIC MUSCLE FORCE AND VOLUNTARY ACTIVATION AS MARKERS OF EXERCISE-INDUCED MUSCLE DAMAGE

4.1.1 INTRODUCTION

The loss of the ability of skeletal muscle to generate force is considered one of the most appropriate and valid means by which to quantify exercise-induced muscle damage (Faulkner *et al.*, 1993; Faulkner and Brooks, 1997; Warren *et al.*, 1999). The most frequently assessed variable is that of a maximal voluntary contraction (MVC) which is typically defined as the maximal muscle force that a highly motivated subject is able to produce voluntarily under particular contractile conditions. The reductions in MVC force associated with damage persist over the entire timescale of the progression of the degenerative and regenerative process i.e. until the muscle returns to its pre-damage condition (Warren *et al.*, 1999). This can not be said of other commonly used markers such as the release of intramuscular proteins into the circulation, soreness and histopathology which are neither observed over the entire time-course nor seen to correlate well with the magnitude of the functional decrements (Warren *et al.*, 1999).

The assessment of skeletal muscle function as a marker of damage necessitates reliable measures of maximal force. Routine measurements of maximal muscle force include many potential sources of error of which the most important may be the possible lack of central drive to the muscles (Merton, 1954; Rutherford *et al.*, 1986; Bulow *et al.*, 1993). The degree of central activation is rarely taken into consideration when assessing maximal muscle force in the research area of exercise-induced muscle damage (for a review see Warren *et al.*, 1999). This makes it difficult to ascertain if any post-exercise reductions in force are actually representative of structural damage to the muscle or muscle fibre integrity or simply a reduction in voluntary drive. It has therefore been recommended that the twitch interpolation technique be routinely employed in muscle damage studies so as to provide an objective assessment of central activation pre- and post-exercise (McArdle and Jackson, 1999; Byrne *et al.*, 2004; Millet and Lepers, 2004).

The twitch interpolation technique involves the delivery of one or more electrical impulses (via nerve, percutaneous or cortical stimulation) to the active muscle during an MVC. The failure of an interpolated twitch to produce an increment in force during an MVC would suggest maximal voluntary activation and maximal force. The presence of a visible twitch, on the other hand, demonstrates that the force produced by the muscle is less than maximal and suggests that either not all motoneurons have been recruited or that they are discharging at sub-optimal firing rates (Todd *et al.*, 2004). Using simple ratios of the size of the superimposed twitch with respect to the size of the same stimulus evoked in a resting muscle (Merton, 1954; Gandevia, 2001) or to the level of voluntary torque (Kent-Braun and Le Blanc, 1996; Knight and Kamen, 2001), the level of voluntary activation can be readily quantified as an interpolated twitch ratio (ITT) or central activation ratio (CAR) respectively. The most appropriate and valid formula for which to quantify activation remains an ongoing research area (Miller *et al.*, 1999; Stackhouse *et al.*, 2000; Behm *et al.*, 2001; Shield and Zhou, 2004).

Although several researchers have examined the reliability of the twitch interpolation technique for the assessment of maximal muscle force and activation, few have focused on the quadriceps muscles (Behm *et al.*, 1996; Norregaard *et al.*, 1997; Oskeui *et al.*, 2003). Furthermore, to the authors' knowledge no studies exist that have formally tested the reliability of maximal quadriceps muscle force and voluntary activation across a timescale (i.e. hours to days) that may be used to characterise the temporal pattern of skeletal muscle damage in response to acute exercise. Such temporal evaluations of reliability estimates are of particular importance given that the reliability of any assessment should always be established with respect to its stated use or 'analytical goal' (Atkinson and Nevill, 1998).

The aim of the present study was to therefore examine the reliability of measurements of maximal isometric quadriceps muscle force and voluntary activation (using the twitch interpolation technique) across a timescale that is typically employed to examine the aetiology of exercise-induced muscle damage. It was intended to provide a baseline data set highlighting the variability of maximal force and activation in a healthy male population free of any exercise-induced muscle damage. Given that estimates of voluntary activation can be affected by type of calculation method

employed (CAR vs ITT), the size of the interpolated twitch and the state (potentiated vs unpotentiated) and size of the resting twitch, the variability associated with each of the previously mentioned variables was also assessed.

4.1.2 METHODS

4.1.2.1 Subjects. Eight healthy active males volunteered to participate in the study (mean ± SD: age 21 ± 1 years; weight 84 ± 8 kg; height 1.8 ± 0.02 m). The study was approved by the Ethics Committee of Liverpool John Moores University and all subjects conformed to the criteria outlined in section 3.1.2.

4.1.2.2 Familiarisation. Subjects underwent extensive familiarisation prior to participating in the reliability study. During such sessions, the subjects were introduced to and familiarised with the procedure of performing isometric MVCs of the quadriceps (4 s duration) with and without twitch interpolation. In the initial session, subjects practiced performing MVCs without twitch interpolation so as to get accustomed to the concept of achieving and maintaining voluntary force. This session was also utilised to obtain maximal current tolerance and establish the supra-maximal current amplitude for superimposition during an MVC. Whilst remaining at rest, the amperage of a 250 V square wave pulse (100 μs, 1Hz) was progressively increased until the point beyond which further increases in intensity caused no further increase in resting twitch force (Newman *et al.*, 2003). In subsequent sessions, subjects alternated between performing MVCs with and without twitch interpolation so that approximately 3-4 trials of each were performed in each session. This approach of alternating between MVCs with and without stimulation was undertaken because many subjects, at this early stage, performed weaker contractions when they were expecting stimulation (presumably because of apprehension of the prospect of receiving noxious stimuli) compared to when they were not expecting stimulation. Familiarisation sessions were completed until subjects' MVC force and voluntary activation demonstrated a plateau effect between sessions. This level of initial consistency was usually achieved within 3 - 7 sessions (mean ± SD: 5 ± 2 sessions)

after which subjects were then considered eligible to participate in formal reliability testing. Familiarisation data from 3 representative subjects who needed varying degrees of familiarisation are displayed in Table 4.1.1.

Table 4.1.1 – MVC force and voluntary activation of 3 representative subjects during the familiarisation period. These subjects needed varying degrees of familiarisation before being considered as eligible to participate in formal reliability testing. Data presented for sessions 2 – 6 are representative of those trials involving electrical stimulation only and correspond to the peak values observed in that session.

		Familiarisation Session					
		1	2	3	4	5	6
Subject 1	MVC Force (N)	720.6	745.5	778.9	802.1	811.3	815.6
	Voluntary Activation (%)	-	87.6	89.1	93.1	96.1	95.9
Subject 2	MVC Force (N)	950.4	975.3	966.8			
	Voluntary Activation (%)	-	97.8	97.2			
Subject 3	MVC Force (N)	729	788.3	838.7	845.1		
	Voluntary Activation (%)	-	91.4	93.5	94.1		

4.1.2.3 Design. Between 1 and 3 days after familiarisation, subjects completed 7 test sessions over a period of 7 days. Each session involved 5 trials of 100% MVC during which twitches were superimposed. The first 3 of these sessions occurred in 1 day (immediately pre- 1 h of a hypothetical exercise bout and 2 h and 6 h post-exercise) with the remaining sessions occurring at 24 h, 48 h, 72 h and 7 days after the hypothetical exercise bout. This design enables measurements of maximal force and voluntary activation to be taken across a timescale for how they are to be assessed in Study 2. A schematic of the experimental procedure is shown in Figure 4.1.1.

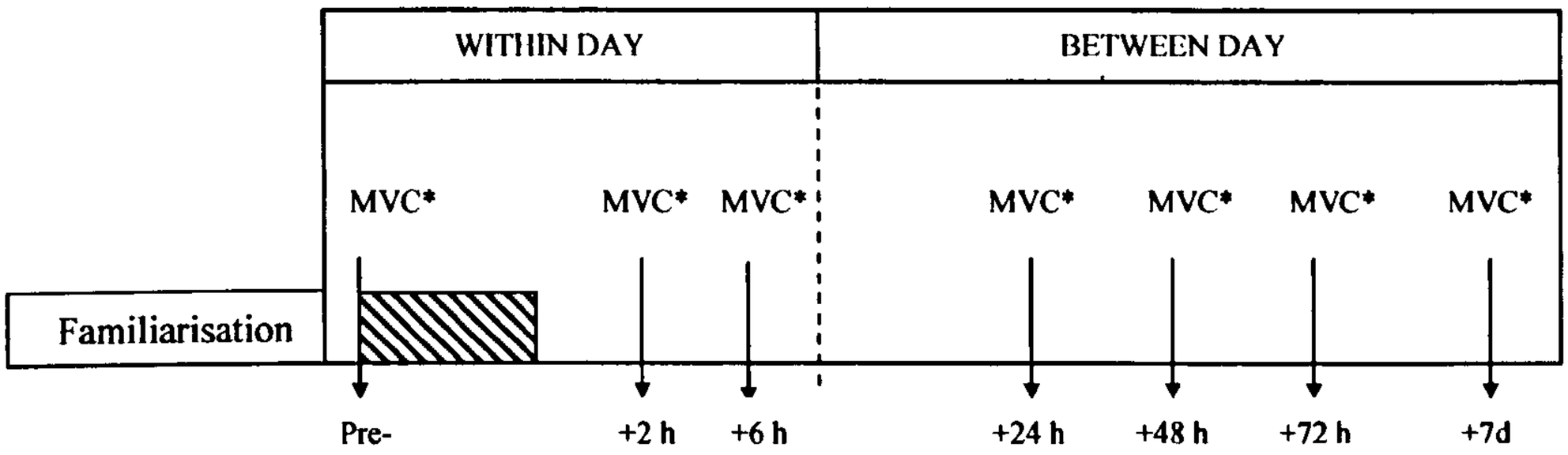
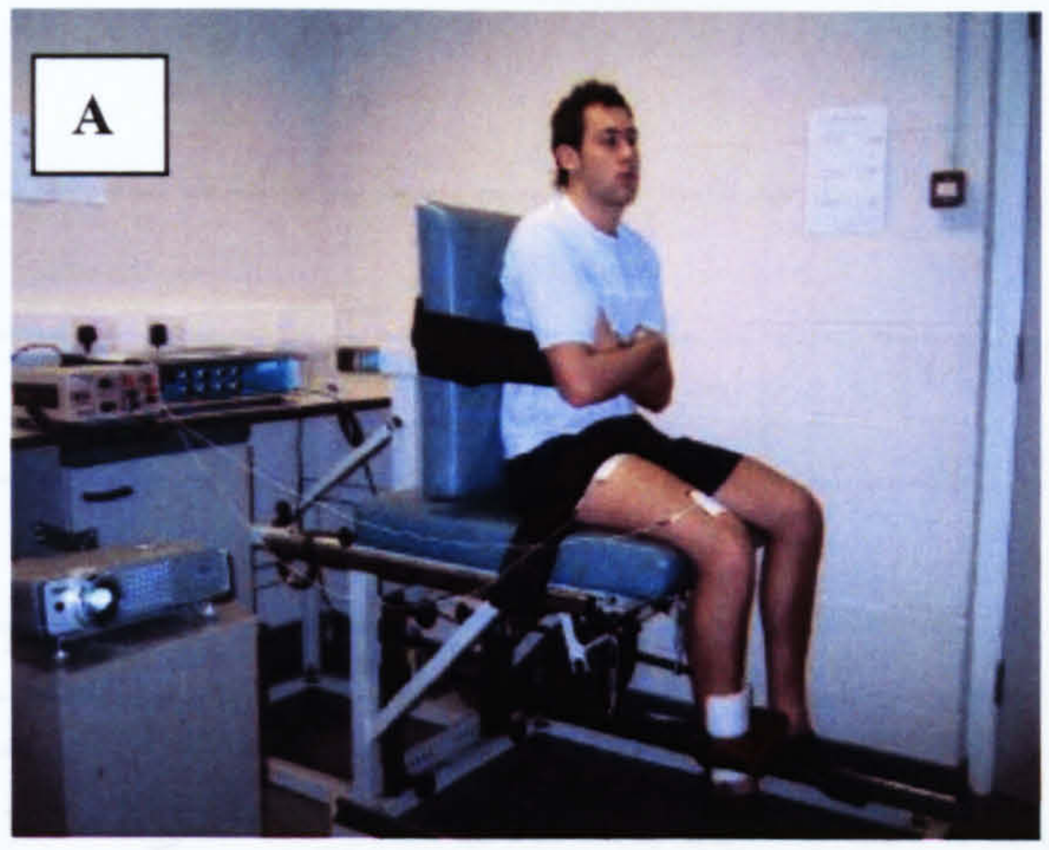


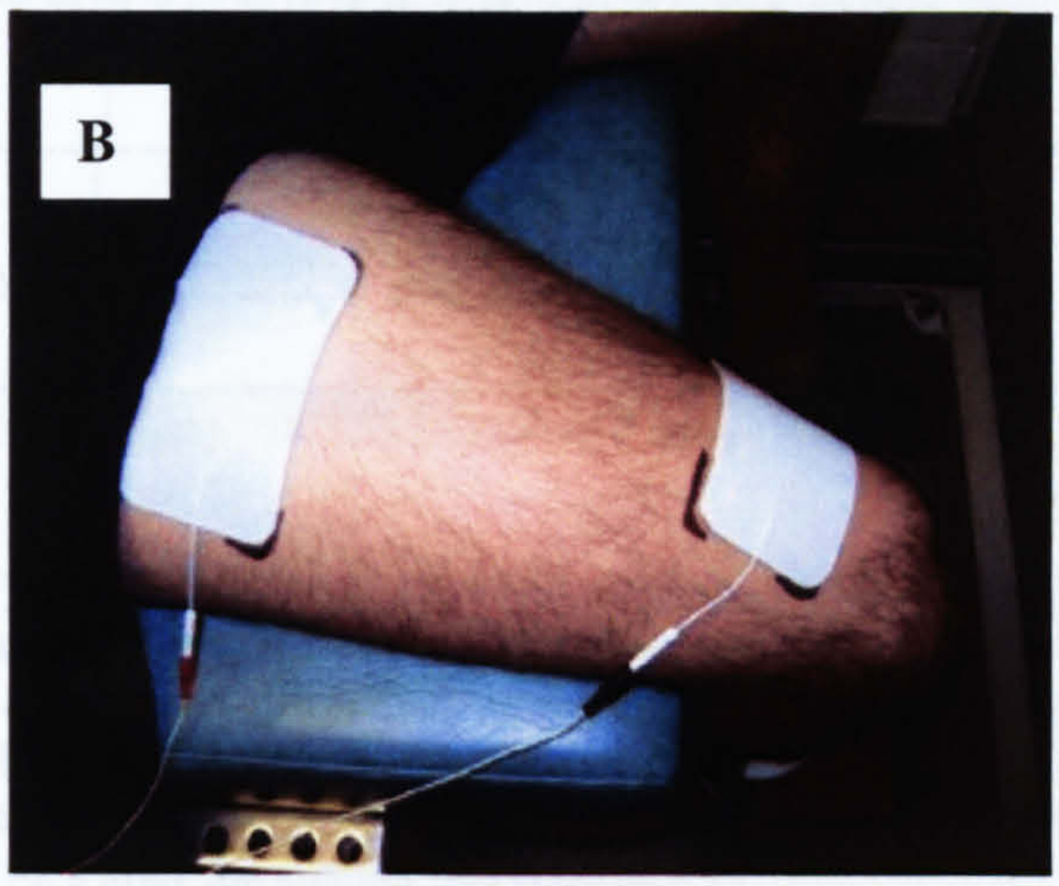
Figure 4.1.1 – Schematic illustration of the experimental design. Shaded area represents 1 h of a hypothetical exercise bout. Each subject performed 5 MVCs with twitch interpolation (*) in each testing session.

4.1.2.4 Maximal isometric quadriceps force. The isometric force of the quadriceps of the subject's dominant leg was measured with the subjects sitting upright in a testing chair. Subjects were seated with the trunk vertical with a 90° flexion in the hip and knee. To prevent extraneous body movements, velcro straps were applied tightly across the thorax and distal thigh. Quadriceps muscle force was measured from the ankle where the attachment was connected to a strain gauge by a metal force transducer (previously calibrated with known weights). After a standard warm-up period, subjects performed 5 trials of 100% MVC (4 s duration) during which supra-maximal twitches were superimposed (see below). A 3-min rest period was included between each trial in an attempt to eliminate the effects of fatigue (Newman *et al.*, 2003). Subjects were given strong verbal encouragement during each trial and visual feedback of their performance was provided during and after each trial via the projection of the computer display onto a large screen placed in front of the subject. The force signal was A/D converted with a sampling frequency of 1000 Hz. Data were acquired for 8 s and analysed with a commercially designed software programme (AcqKnowledge III, Biopac Systems, Massachusetts). An illustration of the experimental set up is displayed in Figure 4.1.2.

4.1.2.5 Twitch Interpolation. The quadriceps were electrically stimulated using two moistened surface electrodes (Chattanooga, USA, 7 cm x 12.7 cm) which were positioned proximally over the vastus lateralis and distally over the vastus medialis.

Skin preparation for each electrode included shaving and light abrasion of the skin followed by cleansing with an isopropyl alcohol swab. A permanent marking pen was used to outline the position of each electrode so as to minimise variability in electrode placement from session to session (Keough *et al.*, 1999). Eight single square wave electrical impulses (100 μs) were delivered during the 8 s sampling period. Each impulse was computer driven and was delivered at 250 V (Digimeter DS7, Hertfordshire, UK). Two impulses were delivered before and after the contraction and allowed comparison of resting twitch amplitudes in an unpotentiated and potentiated condition respectively (Oskouei *et al.*, 2003). The remaining four impulses were delivered during the contraction and tested the maximality of each MVC. The amplitude of supra-maximal superimposed current was that identified for each subject in familiarisation sessions and corresponded to 20% above maximal current tolerance (Todd *et al.*, 2004). An example MVC including the timing of each twitch is provided in Figure 4.1.3.





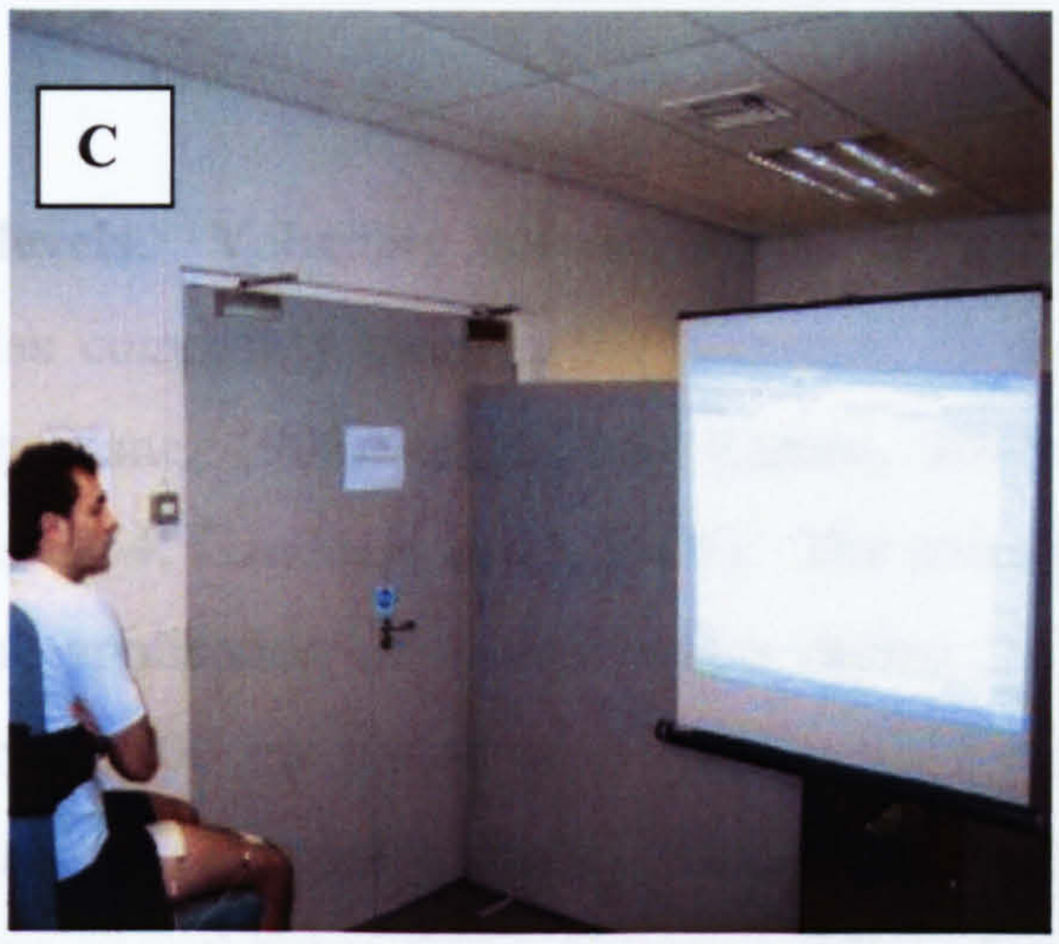


Figure 4.1.2 – Illustration of the experimental set-up. (A) The subject sat with the trunk vertical and a 90° flexion in the hip and knee. (B) Electrodes were placed distally over the vastus medialis and proximally over the vastus lateralis. (C) The subject was provided with real-time visual feedback during each trial.

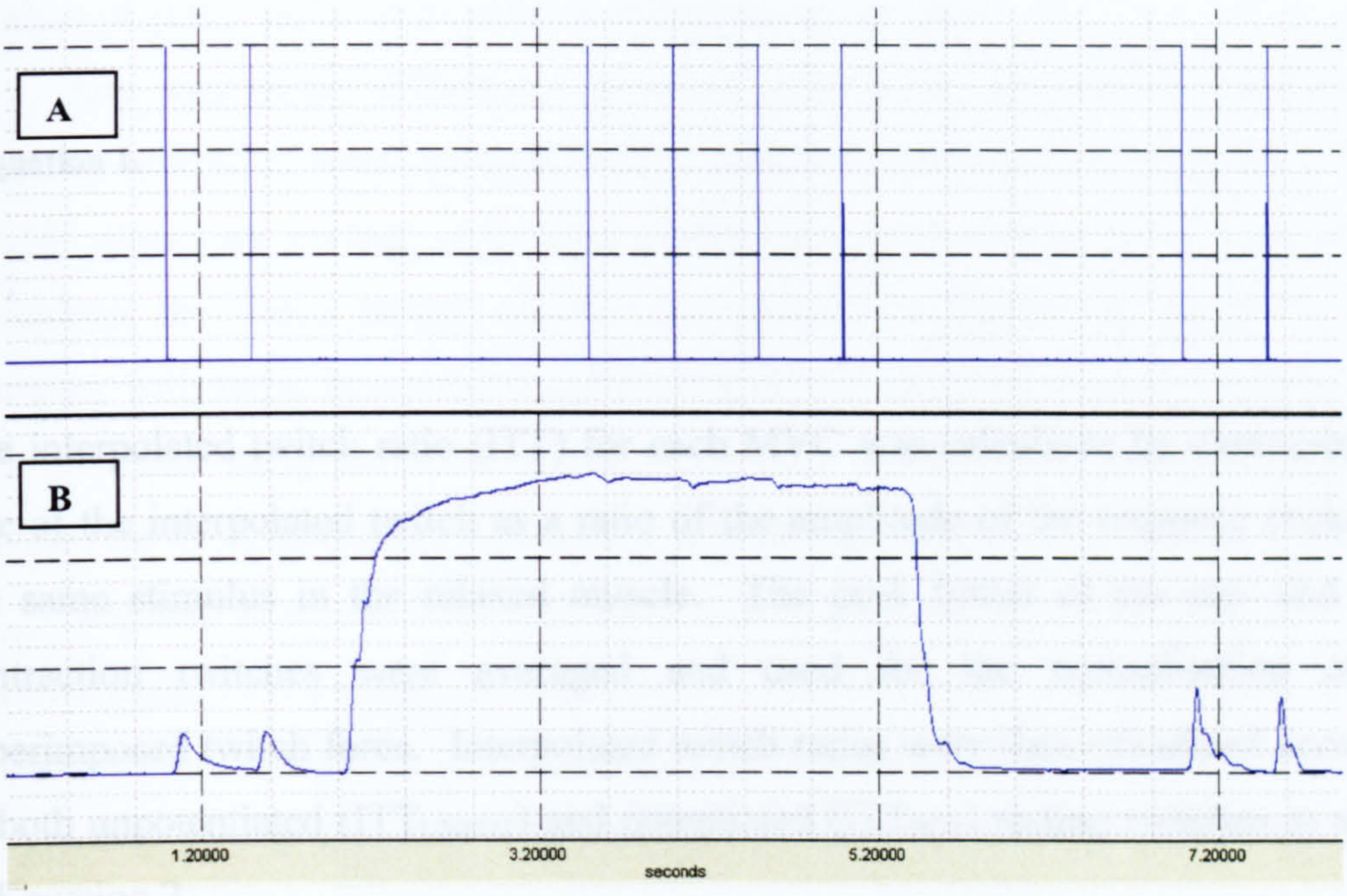


Figure 4.1.3 – Typical MVC with twitches superimposed. Top panel (A) represents timing of superimposed twitches. Two resting twitches were delivered pre- and post-contraction (0.5 s apart) and represented the resting twitch force in an unpotentiated and potentiated state. Four twitches were delivered during the 4 s MVC (0.5 s apart). Bottom panel (B) represents the subject's force-trace. The first potentiated twitch was delivered approximately 1.5 –2 s after the subject had relaxed.

4.1.2.6 Activation levels. Voluntary activation was calculated according to two conventional formulas commonly used in the literature: 1) central activation ratio (Kent-Braun and Le Blanc, 1996; Knight and Kamen, 2001) and 2) interpolated twitch ratio (Merton, 1954; Gandevia *et al.,* 2001). The average force during a 100 ms period before the application of each stimulus during the contraction and the maximal force during a 100 ms period after each stimulus was applied (resulting maximal post-stimulus force) were recorded (Graven-Neilsen *et al.,* 2002). The highest 100 ms mean pre-stimulus force (taken as MVC force) and the resulting maximal post-stimulus force were then subsequently used for analysis and calculation of both central activation ratios and interpolated twitch ratios. The central activation ratio (CAR) for each MVC was calculated as shown in Equation 1:

$$\text{CAR (\%)} = \left[\frac{\text{highest mean pre-stimulus force}}{\text{resulting maximal post-stimulus force}} \right] \text{X } 100$$

Equation 1.

The interpolated twitch ratio (ITT) for each MVC was calculated by expressing the size of the interpolated twitch as a ratio of the amplitude of the response evoked by the same stimulus in the relaxed muscle. The peak forces of the pre- and post-contraction twitches were averaged and used for the normalisation of the superimposed twitch force. Interpolated twitch ratios were then calculated according to both unpotentiated (ITT_{UNPOT}) and potentiated (ITT_{POT}) resting twitches as shown in Equation 2.

$$\text{ITT (\%)} = \left[1 - \left\{ \frac{\text{size of interpolated twitch}}{\text{size of resting twitch}} \right\} \right] \text{X } 100$$

Equation 2.

4.1.2.7 Statistical and data analyses. Mean MVC force, interpolated twitch size, resting twitch size (unpotentiated and potentiated), CAR, ITT_{UNPOT} and ITT_{POT} were recorded for each subject for each session. All data are presented as means ± SD with P values of <0.05 assumed to indicate statistical significance.

Any systematic differences between calculation method on estimates of voluntary activation were assessed using a repeated measures General Linear Model (GLM) where an average activation percentage was calculated for each subject throughout the 7-day testing period. Any systematic differences in MVC force, voluntary activation, interpolated and resting twitch amplitudes across sessions were also analysed using repeated measures GLMs. Ninety-Five percent confidence intervals for the largest mean differences between time points were also calculated.

Random errors between test-times were explored for homoscedasticity (Nevill and Atkinson, 1997) using the predicted vs residuals plot from the GLM. Within-subject standard deviations were calculated (in both absolute terms and as coefficients of variance) as was the 95% "repeatability coefficients" (Bland and Altman, 1999) from $1.96*\sqrt{2*MSE}$ where MSE = mean square error term from the GLM output (Nevill and Atkinson, 1998).

A two-way repeated measurements GLM was used to examine for systematic differences in potentiated and unpotentiated resting twitches over time. Any associations between MVC force and voluntary activation were examined using Spearman's rank correlation.

4.1.3 RESULTS

4.1.3.1 Effects of calculation method on estimates of voluntary activation. The effects of calculation method on estimates of quadriceps voluntary activation are presented in Figure 4.1.4. When using the CAR, ITT_{UNPOT} and ITT_{POT}, estimates of voluntary activation were 98.9 ± 0.8, 91.2 ± 6.0 and 94.6 ± 4.1 % respectively. There was a significant difference between calculation method on estimates of voluntary activation ($P<0.05$) which was present between all pair-wise comparisons.

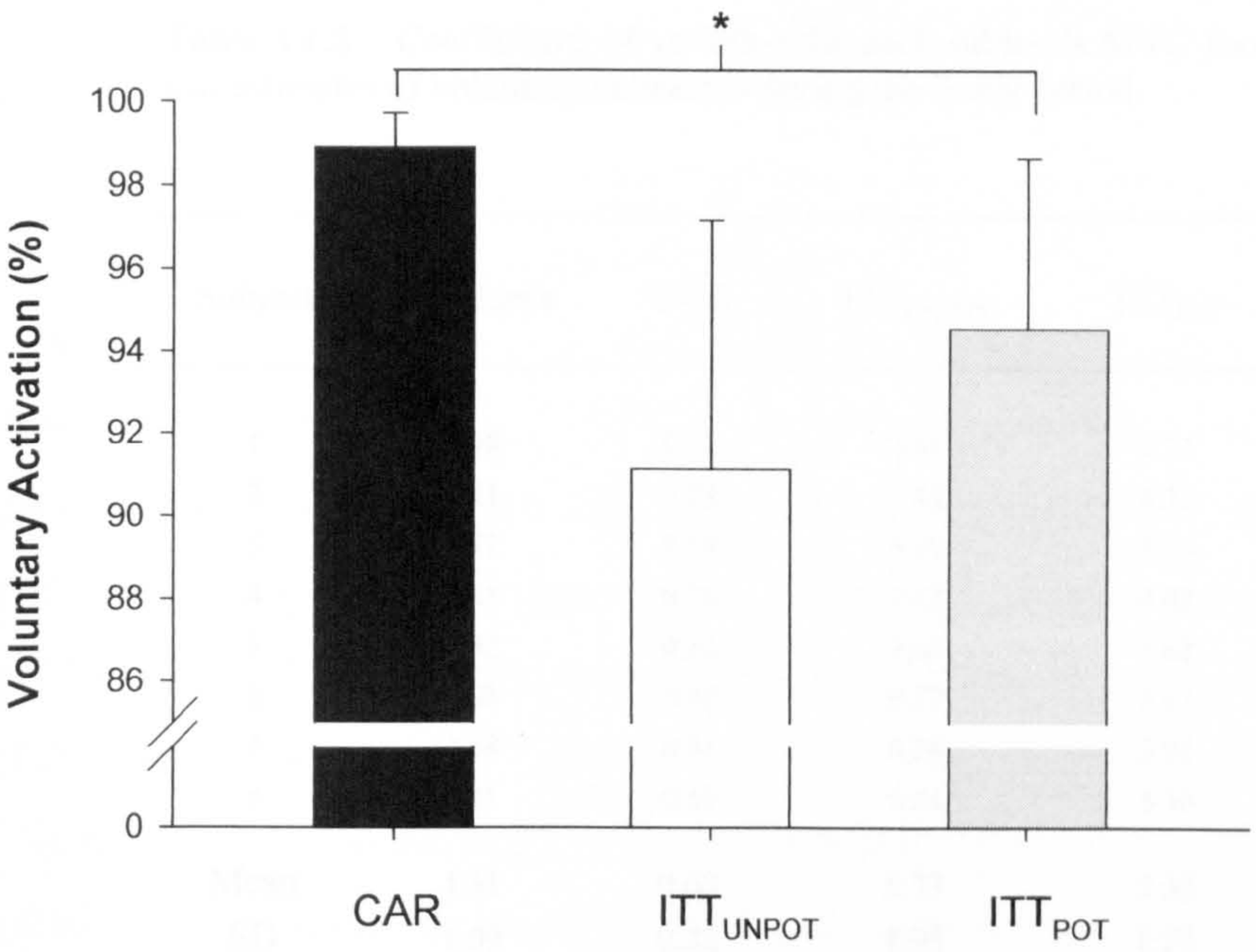


Figure 4.1.4 – The effects of calculation method on estimates of voluntary activation. * denotes significant difference between all pair-wise comparisons, $P<0.05$.

4.1.3.2 Intra-subject variability in MVC force and activation. Coefficients of variations (CVs) for each individual over the 7-day period are presented in Table 4.1.2. Individual CVs for MVC force ranged from 2.98 to 6.52% and the mean CV for the group of subjects was 4.31 ± 1.09%. With regard to activation formula, both individual and mean CV of the ITT_{POT} showed the greatest association with the variability in MVC force whereas the CAR showed the least. At first glance, these

data seem to indicate that the CAR is the most reliable estimate of voluntary activation. However, because of its consistent overestimation, CAR values did not change in accordance with small fluctuations in voluntary force. Thus, the CAR not only appears to result in overestimations of voluntary activation but is also not sensitive enough to discriminate between small fluctuations in voluntary force which should correspond to increases or decreases in voluntary activation accordingly. The ITT_{POT} therefore appears to provide the most valid estimation of voluntary activation and hence all estimates of voluntary activation presented hereafter refer to the ITT_{POT} method.

Table 4.1.2 – Coefficients of variation for each subject's MVC force and estimates of voluntary activation during the 7-day period.

Subject	MVC force	CAR	ITT_{UNPOT}	ITT_{POT}
1	2.98	0.31	3.54	1.77
2	4.41	0.74	7.43	4.32
3	4.72	0.59	5.19	3.19
4	4.68	0.76	7.12	4.48
5	6.52	0.32	3.04	1.62
6	3.98	0.93	8.77	5.27
7	3.68	0.81	5.24	3.01
8	3.41	0.59	5.54	3.36
Mean	4.31	0.63	5.73	3.38
SD	1.09	0.22	1.95	1.28

4.1.3.3 Reliability of MVC force and voluntary activation. The variability in MVC force and activation across sessions is presented in Figure 4.1.5. There was no significant change in MVC force across the 7 sessions ($P>0.05$) indicating that no significant learning effect had occurred. The largest mean difference between any test sessions was 39.8 N (95% confidence intervals: 18.6 – 61.0 N). The 95% repeatability coefficient for MVC force was ± 76.03 N. This statistic estimates that in individual subjects who display no symptoms of exercise-induced muscle damage,

95% of the differences in MVC force measured in repeated trials across 7 days will not exceed or be less than 76.03 N.

In accordance with MVC force, voluntary activation also did not significantly change during the 7 sessions ($P>0.05$). The largest mean difference between any test sessions was 1.4% (95% confidence intervals: 1.2 – 4.1%). The 95% repeatability coefficient for voluntary activation throughout the 7 sessions was ± 4.42%.

4.1.3.4 Interpolated and resting twitch sizes. The variability in the size of the interpolated twitch across sessions is presented in Figure 4.1.6. Although not significant, there was a tendency for the interpolated twitch force to change throughout the 7 sessions ($P = 0.063$). The largest mean difference between any test sessions was 4.6 N (95% confidence intervals: 0.1 – 9.2 N). The 95% repeatability coefficient for the interpolated twitch size was ± 8.44 N. The variability in resting twitch size across sessions is presented in Figure 4.1.7.

As expected, there was a significant difference between potentiated and unpotentiated resting twitches ($P<0.05$). On average, potentiated and unpotentiated twitches corresponded to 19.58 ± 3.17 and 12.03 ± 1.66 % of MVC force. There was no significant change in either unpotentiated or potentiated resting twitches over the 7 days ($P>0.05$). The largest mean difference between any test sessions for unpotentiated and potentiated twitches was 11.0 N (95% confidence intervals: 3.6 – 18.5 N) and 17.7 N respectively (95% confidence intervals: 4.4 – 39.9 N). The 95% repeatability coeffcients for unpotentiated and potentiated resting twitches were ± 25.92 N and ± 43.58 N respectively.

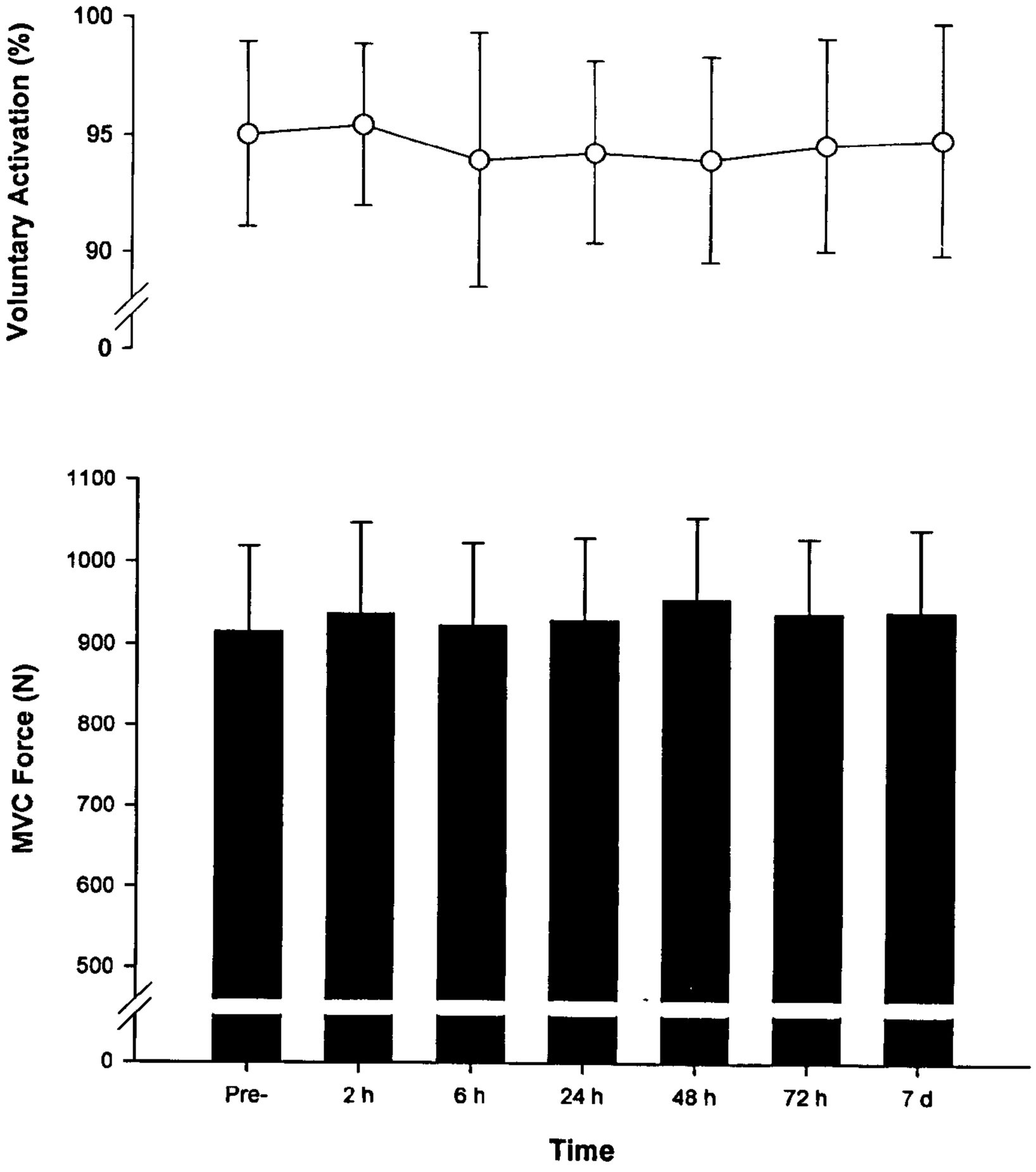


Figure 4.1.5 – MVC force and voluntary activation during the 7-day testing period.

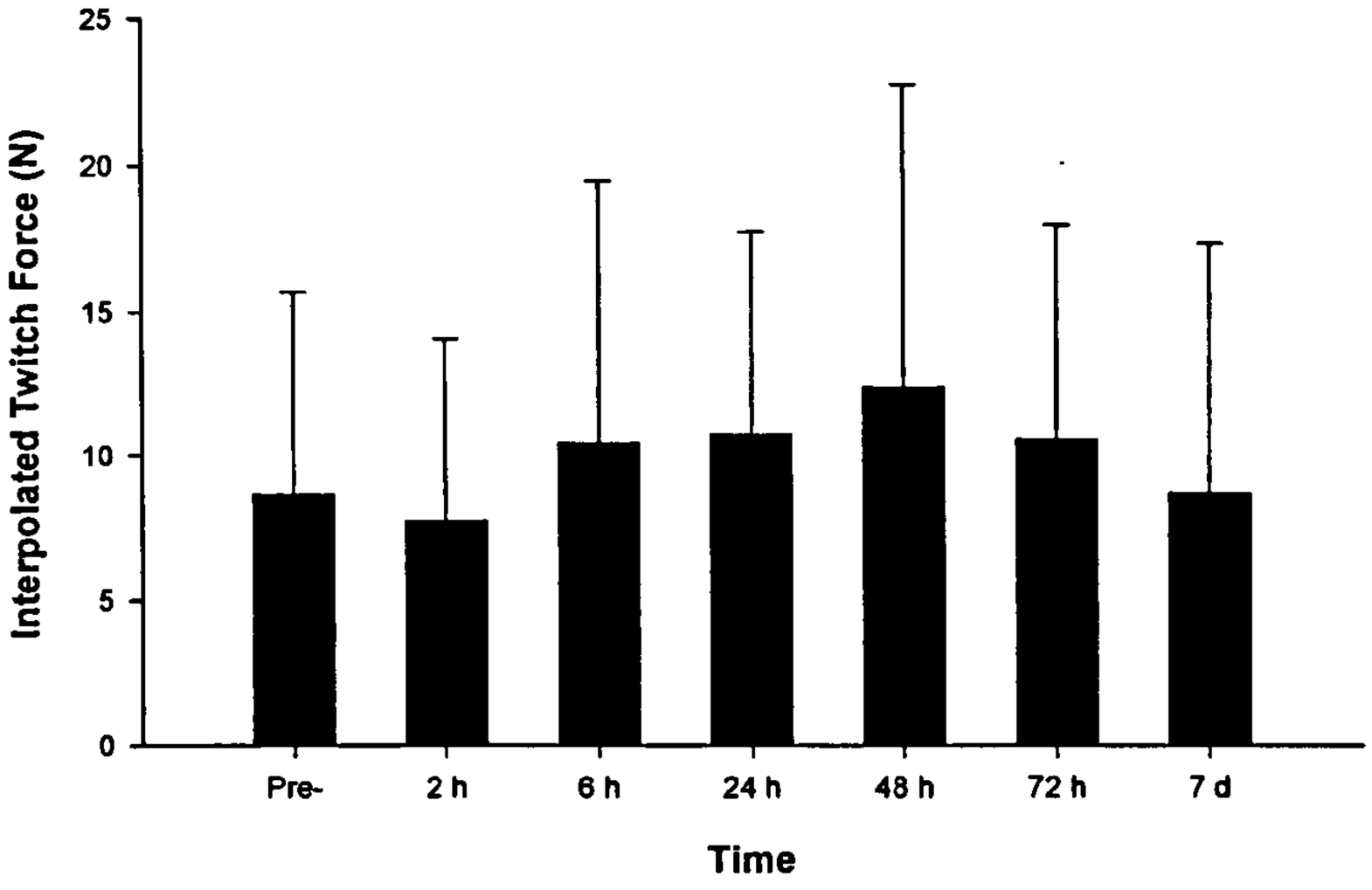


Figure 4.1.6 – Variations in the interpolated twitch force across sessions.

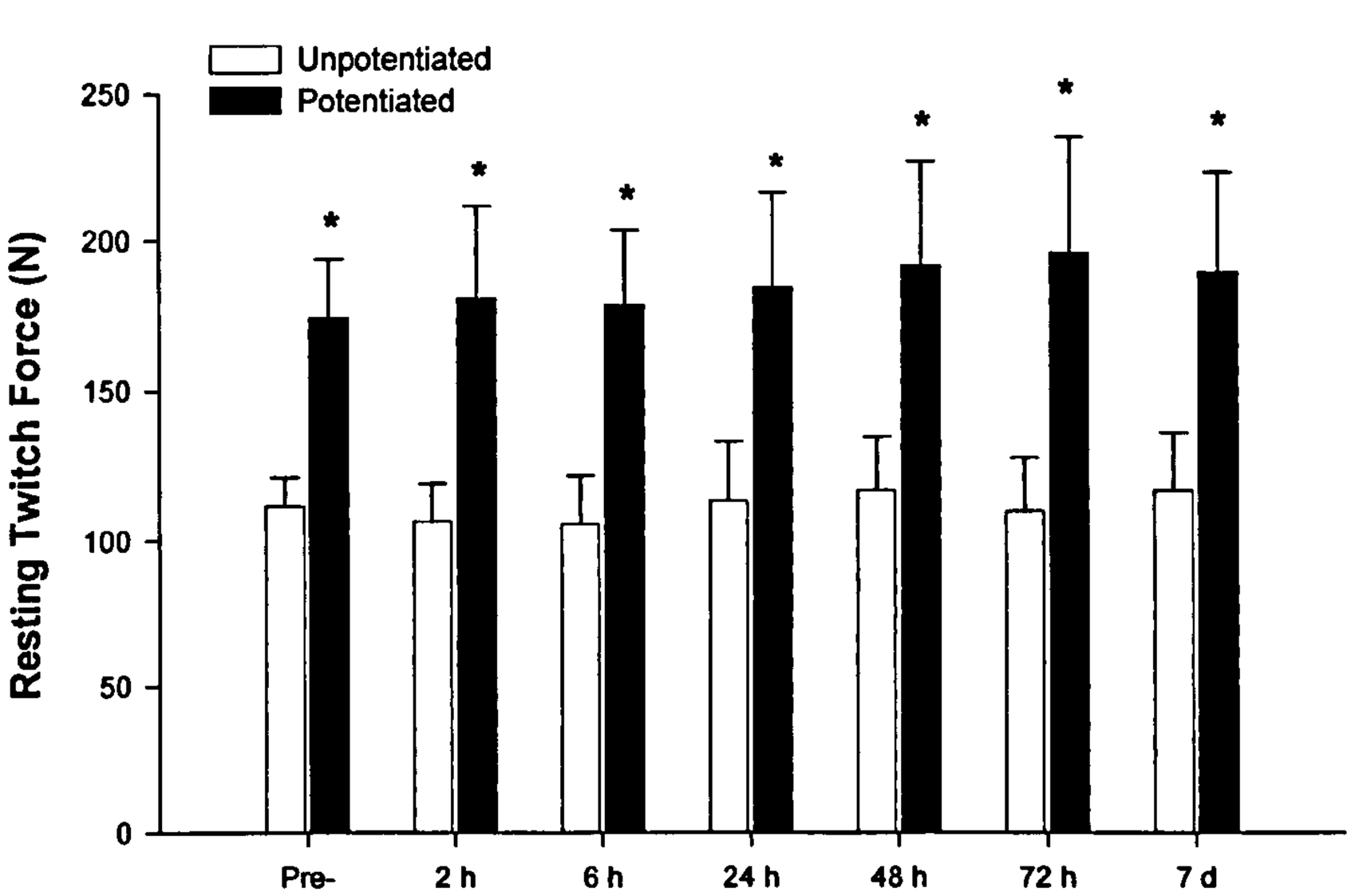


Figure 4.1.7 – Effects of potentiation on resting twitch force across sessions. * denotes significant difference between potentiated and unpotentiated twitches at all time points, $P<0.05$.

4.1.3 DISCUSSION

The aim of the present study was to establish the reliability of maximal isometric quadriceps muscle force and voluntary activation across a timescale for how these variables are to be assessed in Study 2. To the authors' knowledge, this is the first study to conduct such a temporal evaluation of reliability estimates. Given that estimates of voluntary activation can be affected by type of calculation method, the size of the interpolated twitch and the size and nature of the resting twitch, the variability associated with such variables was also assessed.

Method of calculation. When using an ITT ratio, the control twitch is usually produced in a relaxed potentiated muscle, a short time (<5 s) after the maximal effort (Gandevia, 2001; Shield and Zhou, 2004). The present data reveals, at least for single stimuli, that failure to scale the superimposed twitch to a potentiated resting twitch may result in an underestimation of voluntary activation of approximately 3%. It is thus recommended that control twitches are delivered both pre- and post-contraction so as to ensure that the control twitch is potentiated.

The present data also suggest that the CAR may overestimate central activation by approximately 4% than when compared to the ITT_{POT}. This result is of considerable importance considering that previous studies have also used the CAR as a measure of central activation as calculated from a single superimposed stimulus (e.g. Knight and Kamen, 2001). These results compare favourably with those of Behm *et al.* (2001) who concluded for doublet stimulations that CAR estimates of voluntary activation for the quadriceps are 1.9% higher than interpolated twitch ratios.

It is acknowledged that CAR values would have been expected to be lower if multiple stimuli had been delivered (Strojnik *et al.*, 1995; Kent-Braun and Le Blanc, 1996; Miller *et al.*, 1999). This approach, however, is often problematic due to antidromic activation of motoneurons and Renshaw cells plus reflex effects on synergists and remote muscles (Gandevia, 2001). Single impulses, on the other hand, produce brief force transients which are likely to be minimally distorted by spinal reflexes (Herbert *et al.*, 1997). Recent reports have also suggested, at least for maximal contractions of the quadriceps, that there are no differences in estimates of

voluntary activation (as calculated from interpolated twitch ratios) when single or multiple stimuli are superimposed (Shield and Zhou, 2004).

The present data also revealed coefficient of variations of 0.63, 3.38 and 4.31% for the CAR, ITT_{POT} and MVC force respectively. At first glance, these data seem to indicate that the CAR is the most reliable estimate of voluntary activation. However, because of its consistent overestimation, CAR values did not change in accordance with small fluctuations in voluntary force. Thus, not only does the CAR result in overestimations of voluntary activation but it also appears that it is non-responsive to small fluctuations in voluntary force which should correspond to increases or decreases in voluntary activation accordingly.

In terms of quantifying activation and in accordance with other authors (Herbert *et al.*, 1997; Gandevia, 2001; Shield and Zhou, 2004), it is recommended that the use of the more conventional interpolated twitch ratio be employed whereby the size of a single superimposed twitch is scaled to the size of the same stimulus delivered to a resting potentiated muscle.

Reliability of maximal force and voluntary activation. There was no evidence of any systematic bias in maximal quadriceps force or voluntary activation during the 7-day testing period i.e. no significant 'learning effect' occurred. The present data therefore indicate that well-familiarised subjects are capable of reproducing their perceived maximal efforts both within day and between days.

The 95% repeatability coefficient for the repeated measurements of MVC force and voluntary activation were ± 76.03 N and ± 4.42% respectively. These statistics estimate that in individual subjects who display no symptoms of exercise-induced muscle damage, 95% of the differences measured in repeated trials during 7 days will not exceed or be less than these values. Providing activation remains within these limits in the days following an acute bout of exercise, the researcher would be reasonably certain (95%) that muscle damage is present if MVC force falls outside these limits. This of course can then be confirmed by other indirect markers of damage such as the release of intramuscular proteins into the circulation and subjective feelings of pain and soreness.

The average voluntary activation percentage (ITT_{POT}) during the 7 days was 94.6 ± 4.1% with individual values ranging from 86.03 to 97.41%. Only in 21 of the

total 280 maximal attempts was the interpolated twitch completely occluded (i.e. full activation achieved). These values compare well with previous studies where activation levels of 85-95% have typically been reported (Suter *et al.*, 1996; Shield and Zhou, 2004). It is now well documented that in comparison with other muscles, quadriceps activation is substantially harder to achieve (Behm *et al.*, 2002). The finding is likely explained by the size principle of motor recruitment (Henneman *et al.*, 1974) whereby the predominantly fast twitch knee extensors (Gollnick *et al.*, 1973) would be more difficult to activate given the higher recruitment threshold of fast twitch motor neurons.

In accordance with the recent views of Todd *et al.* (2004), the author is also of the opinion that several important experimental factors underlie the highly reliable nature of the present data. These include the extensive familiarisation period that each subject undertook prior to participating in formal reliability testing, the use of real-time visual feedback of each subject's force production, the provision of subjects with strong verbal encouragement during each contraction and also the use of a quiet and private room to conduct all testing sessions. All of these factors can contribute to subjects' motivation levels which are of paramount importance in achieving reliable and valid maximal efforts.

Variability of the interpolated and resting forces. Although not significant, there was a tendency for the interpolated twitch force to vary throughout the 7 days. This finding is not novel and has also been recently reported for near maximal contractions of the quadriceps (Oskouei *et al.*, 2003). The present extend the previous findings by highlighting the variability in interpolated twitch force over a much larger sampling period and have quantified this using the 95% repeatability coeffcient where a value of 8.4 N was calculated.

Oskouei *et al.* (2003) described their finding as 'disappointing' given that maximal contractions are the most frequently used contractions to assess motor function in both the clinical and applied research setting. These authors have no ready explanation for the variations in superimposed twitch force that is present in maximal contractions. The present data also provide no insight as to what factors may have caused these variations. It appears that variations in the superimposed twitch force exist that are therefore inherent (i.e. random error) of the twitch

interpolation technique particularly for maximal or near maximal (>90%) contractions of the quadriceps.

As expected, there was a significant difference in potentiated and unpotentiated twitch forces. Specifically, potentiated and unpotentiated twitches corresponded to 19.58 ± 3.17 and 12.03 ± 1.66% of MVC force respectively. Although neither twitch force significantly changed during the 7 days, potentiated twitches appeared to be somewhat more variable than unpotentiated twitches (95% repeatability coefficients; ± 43.58 N vs ± 25.92 N). The enhanced variability associated with potentiated twitches is most likely related to timing of twitches. The potentiation effect, although apparent for minutes, decreases very quickly after relaxation and can change by as much as up to 50% in the first 10 second after deactivation (Bulow *et al.*, 1993). In the present study, potentiated twitches were delivered at approximately 1.5 and 2 s after the subject had relaxed. However, this time interval was by no means exact given that individuals displayed different rates of relaxation that also varied both within session and between sessions (results not shown). It is therefore recommended that special attention be given to the timing of potentiated twitches so as to ensure that each twitch is delivered as precisely as possible after deactivation.

In summary, it is concluded that young healthy well-familiarised male subjects can perform reliable and reproducible measures of maximal quadriceps force and voluntary activation across a timescale that is typically used to examine the temporal pattern of exercise-induced muscle damage. The variability associated with voluntary activation and maximal force (in individual subjects who are free of any symptoms of exercise-induced muscle damage) can be estimated in healthy young males using the 95% repeatability coefficient where values of ± 4.42% and ± 76.03 N were observed respectively. Such a population can routinely achieve activation levels (ITT_{POT}) of >90% although full voluntary activation is rarely achieved in either single or repeated trials. The twitch interpolation technique will therefore be employed in Study 2 so as to ensure that any observed post-exercise reductions in voluntary force are not simply a reflection of a reduced central drive.

4.2 THE DEVELOPMENT OF A NON-DAMAGING TREADMILL EXERCISE PROTOCOL AS A STIMULUS TO INITIATE THE EXERCISE-INDUCED EXPRESSION OF HSPS IN HUMAN SKELETAL MUSCLE

4.2.1 INTRODUCTION

Interpretation of HSP data from human exercise studies is complicated by the variations in timing of tissue sampling between studies and perhaps more importantly, the disparate exercise protocols utilised by investigators. This is particularly the case in those instances where there is a damaging component to the exercise protocol, where damage is defined as gross necrosis and a significant reduction in the force generating capability of the muscle (Vasilaki *et al.*, 2006). Following damage to skeletal muscle fibres, there is a subsequent inflammatory response that is characterised by the invasion of neutrophils, macrophages and inflammatory mediators into the cell (MacIntyre, 1995; Clarkson and Sayers, 1999). The inflammatory response is believed to be responsible for the 'secondary damage' phenomenon (damage occurring several days post-exercise that follows the initial mechanical disruptions) caused by neutrophil derived reactive oxygen species (ROS). The elevated levels of neutrophils in the muscle release proteolytic enzymes and oxygen radicals that assist in the degradation of damaged tissue and removal of cellular debris (Clarkson and Hubal, 2002).

Exercise protocols that involve a damaging component complicate our understanding of the cellular stress response to exercise because phagocytic cells which migrate to the site of injury contain relatively high levels of HSPs (Khassaf *et al.*, 2003). The invasion of phagocytic cells would also result in neutrophil derived increases in ROS that may further augment the intra-muscular expression of HSPs. Analysis of muscle homogenates obtained from muscles that have undergone exercise protocols that are damaging in nature would therefore make it extremely difficult to specifically quantify those HSPs that are induced by skeletal muscle cells by factors occurring during exercise (i.e. increased temperature etc) or are merely due to changes in phagocytic cell content (Vasilaki *et al.*, 2006). The use of a non-damaging exercise protocol (where non-damaging is defined as exercise that induces no overt structural or functional damage to the muscle) provides a more controlled methodological

approach whereby the increased expression of HSPs is likely to have arisen from skeletal muscle cells rather than gross changes in phagocytic cell content (Vasilaki *et al.*, 2006).

In evaluating the extent of contraction-induced damage associated with a particular exercise protocol, many authors have solely relied on an evaluation of post-exercise circulating creatine kinase levels (Walsh *et al.*, 2001; Khassaf *et al.*, 2001; Febbraio *et al.*, 2002a). Given that the loss of the ability of the muscle to generate force is considered the most valid and reliable marker of exercise-induced muscle damage (Warren *et al.*, 1999; Clarkson and Hubal, 2002), it would therefore seem appropriate to also evaluate maximal muscle force pre- and post-exercise before describing a chosen exercise protocol as non-damaging. Furthermore, considering that the magnitude of exercise-induced muscle damage is strongly influenced by training status and prior exposure to the task (Morgan and Allen, 1999; Proske and Morgan, 2001), it is also important to consider individual variations in physical fitness before concluding that a particular exercise bout is non-damaging. The degree of damage associated with a chosen exercise protocol should therefore be evaluated for the specific population (s) under consideration.

The aim of the present study was to therefore develop a non-damaging treadmill exercise protocol to utilise in all exercise related studies as a stimulus to initiate the exercise-induced expression of HSPs in human skeletal muscle. Given that the magnitude of muscle damage can be influenced by training status (Proske and Morgan, 2001), both active and trained individuals were included in the subject population. Running was chosen as the exercise mode because available data concerning HSP expression in response to treadmill exercise protocols are extremely limited. Furthermore, running is also the most widely used form of 'keep-fit' activity in the general population and is the main activity involved in an array of sports.

4.2.2 METHODS

4.2.2.1 Subjects. Twelve healthy young males volunteered to participate in the study (mean ± SD: age, 25 ± 4 years; weight, 75 ± 8 kg; height, 1.77 ± 0.05 m). The study was approved by the Ethics Committee of Liverpool John Moores University and all subjects conformed to the criteria outlined in section 3.1.2.

4.2.2.2 Design. All subjects were initially assessed for $\dot{V}O_{2max}$ (see section 3.3) and lactate threshold (see section 3.4). These tests were separated by at least 48 h. Subjects were subsequently divided into an 'active' (n=6) or 'trained' (n=6) group according to the criteria outlined in section 3.1.2. Having then refrained from exercise and prolonged thermal exposure (i.e. baths, saunas, steam rooms, tanning devices etc) for 3-5 days following the lactate threshold test, all subjects completed a 45 min running exercise protocol on a motorised treadmill at a speed corresponding to their lactate threshold (see section 3.8). Ratings of perceived muscle soreness (see section 3.5), maximal isometric quadriceps muscle force and voluntary activation were assessed immediately before and at 2 h, 6 h, 24 h, 48 h, 72 h and 7 days post-exercise (see section 4.1.2). Venous blood samples were also obtained at these time-points and analysed for serum creatine kinase (CK) activity (see section 3.9 and 3.13). Two subjects (1 trained and 1 active) also completed the exercise protocol 6-8 weeks later to provide pilot data assessing if the exercise bout was sufficient to induce HSP expression. Muscle biopsies were obtained from the vastus lateralis (see section 3.10) immediately prior to exercise and at 48 h post-exercise and analysed for HSP70 and HSP60 content (see section 3.11).

4.2.2.3 Statistical analyses. Differences in baseline physical and physiological characteristics between groups were analysed using students *t*-test for independent samples. Changes in exercise related variables during the exercise protocol were analysed using a two-way mixed design General Linear Model where the 'within subjects' factor was time and 'between subjects' factor was training status. Similarly, changes in MVC force, voluntary activation, CK activity, pain diagram and VAS scores pre- and post-exercise were also analysed using a two-way mixed design GLM. Where there was a significant main effect for time, paired *t*- tests with Bonferroni

corrections were used for post-hoc analysis. All data are presented as means ± SD with P values of <0.05 indicating statistical significance.

4.2.3 RESULTS

4.2.3.1 Baseline physical and physiological characteristics. Physical and physiological baseline characteristics of the active and trained subject groups are displayed in Table 4.2.1. There was a significant difference (P<0.05) between groups in $\dot{V}O_{2max}$, lactate threshold (both % of $\dot{V}O_{2max}$ and running speed at LT) and number of hours involved in physical activity per week.

Table 4.2.1 – Physical and physiological characteristics of the active and trained subject groups.

	ACTIVE	TRAINED
Age (years)	25 ± 2	25 ± 5
Height (m)	1.78 ± 0.04	1.74 ± 0.06
Weight (kg)	76.3 ± 7.6	74.3 ± 7.9
$\dot{V}O_{2max}$ ($ml.kg^{-1}.min^{-1}$)	56.4 ± 3.3	66.4 ± 5.4 *
LT (% of $\dot{V}O_{2max}$)	69.3 ± 2.1	76.8 ± 1.4 *
Running speed @ LT ($km.h^{-1}$)	11.2 ± 0.7	13 ± 0.9 *
Hours of activity per week	2.9 ± 1.1	7.5 ± 1.5 *

* denotes significant difference between groups, P<0.05

4.2.3.2 Physiological responses to the exercise protocol. Subjects' heart rates and blood lactate concentration during exercise are shown in Figure 4.2.1 and Figure 4.2.2, respectively. Heart rate showed a significant increase (P<0.05) during exercise in both groups. There was no significant difference (P>0.05) in heart rate between groups at any time point during exercise. There was no significant change in blood lactate during exercise in either group (P>0.05) and there was also no significant

difference in blood lactate between active and trained subjects at any time point during exercise (P>0.05). Although both groups were running at a similar intensity in terms of lactate threshold, the trained subjects were running at a significantly higher (P<0.05) intensity relative to $\dot{V}O_{2max}$ (Figure 4.2.3). There was no significant change in exercise intensity relative to $\dot{V}O_{2max}$ during exercise in either group (P>0.05). Subjects' RPE showed a progressive significant increase (P<0.05) during exercise in both groups (Figure 4.2.4). There was no significant difference in RPE between active and trained subjects at any time point during exercise (P>0.05).

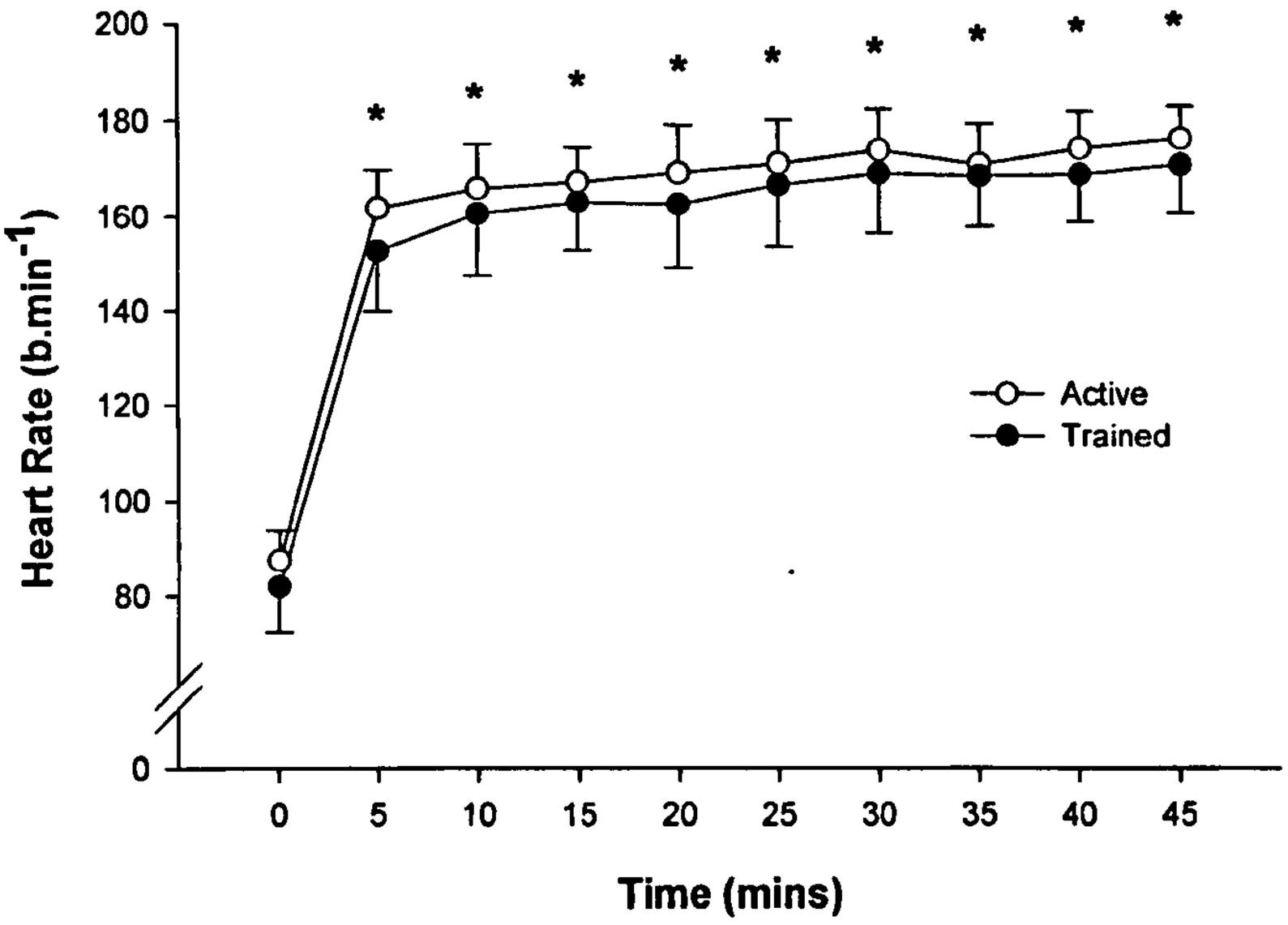


Figure 4.2.1 – Heart rates of the active and trained subjects during the exercise protocol. * denotes significant difference from pre-exercise values, P<0.05.

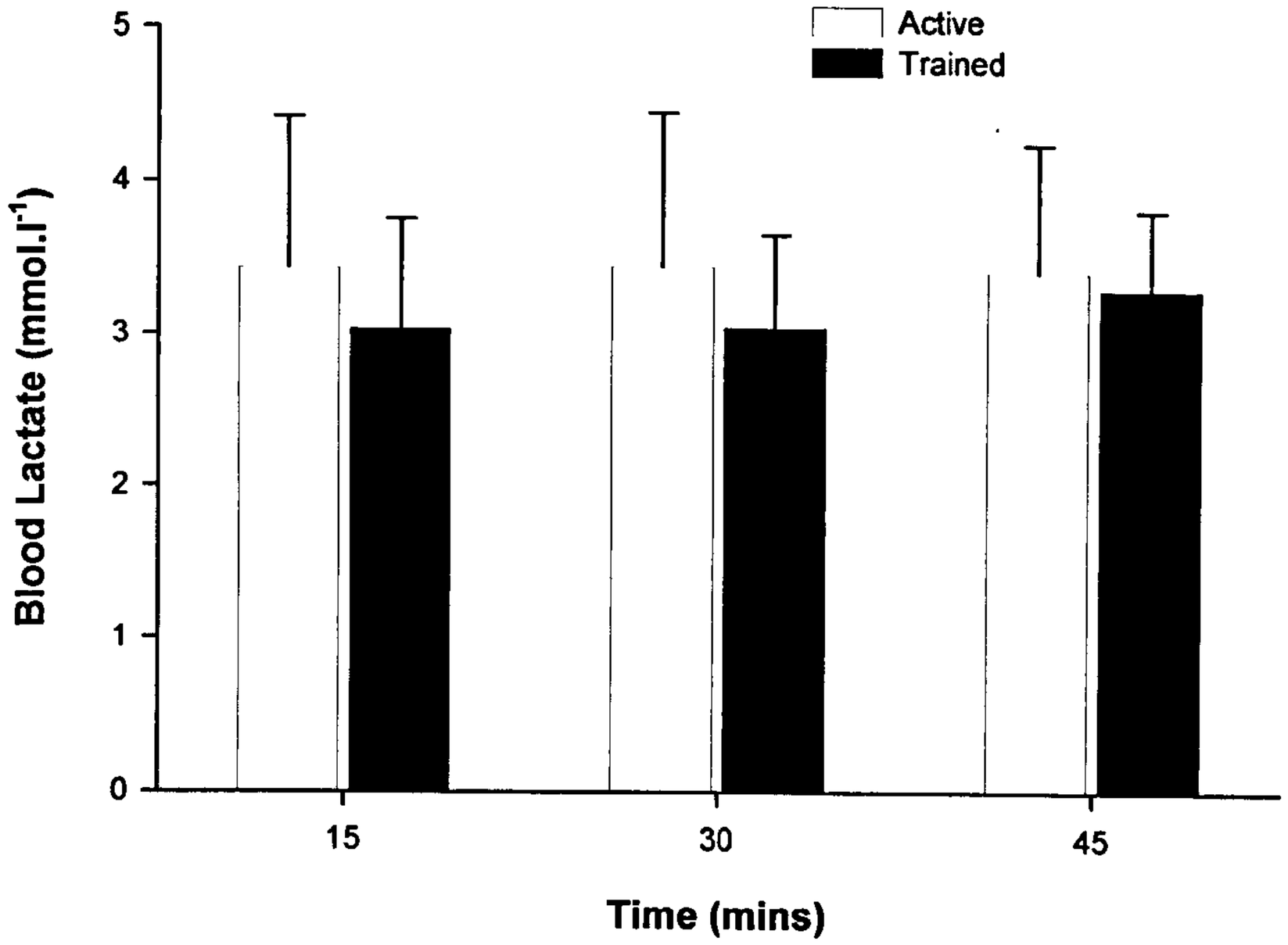


Figure 4.2.2 – Blood lactate concentrations of the active and trained subjects during the exercise protocol.

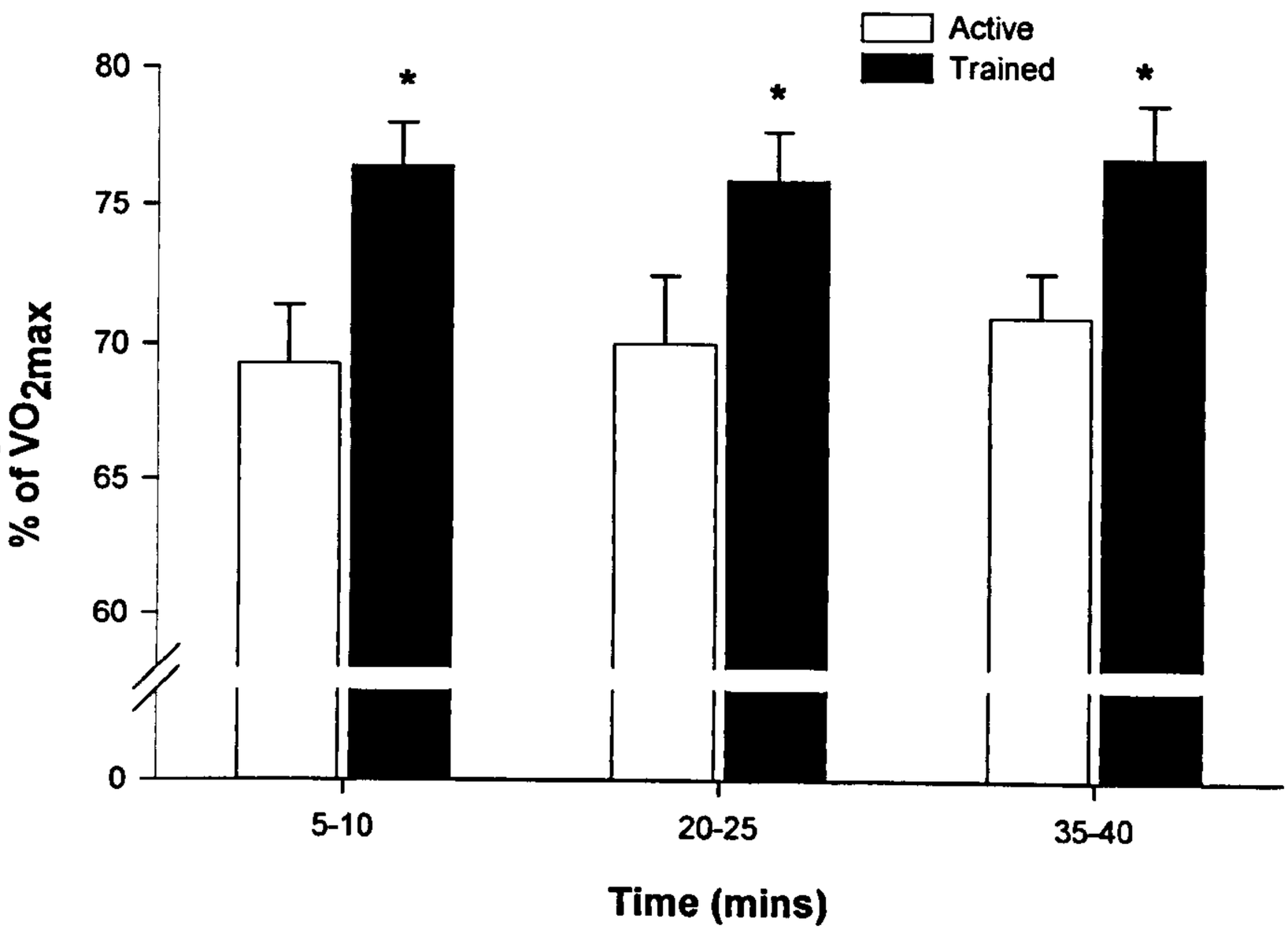


Figure 4.2.3 – Exercise intensity, relative to $\dot{V}O_{2max}$, of the active and trained subjects during the exercise protocol. * denotes significant difference between groups, $P<0.05$.

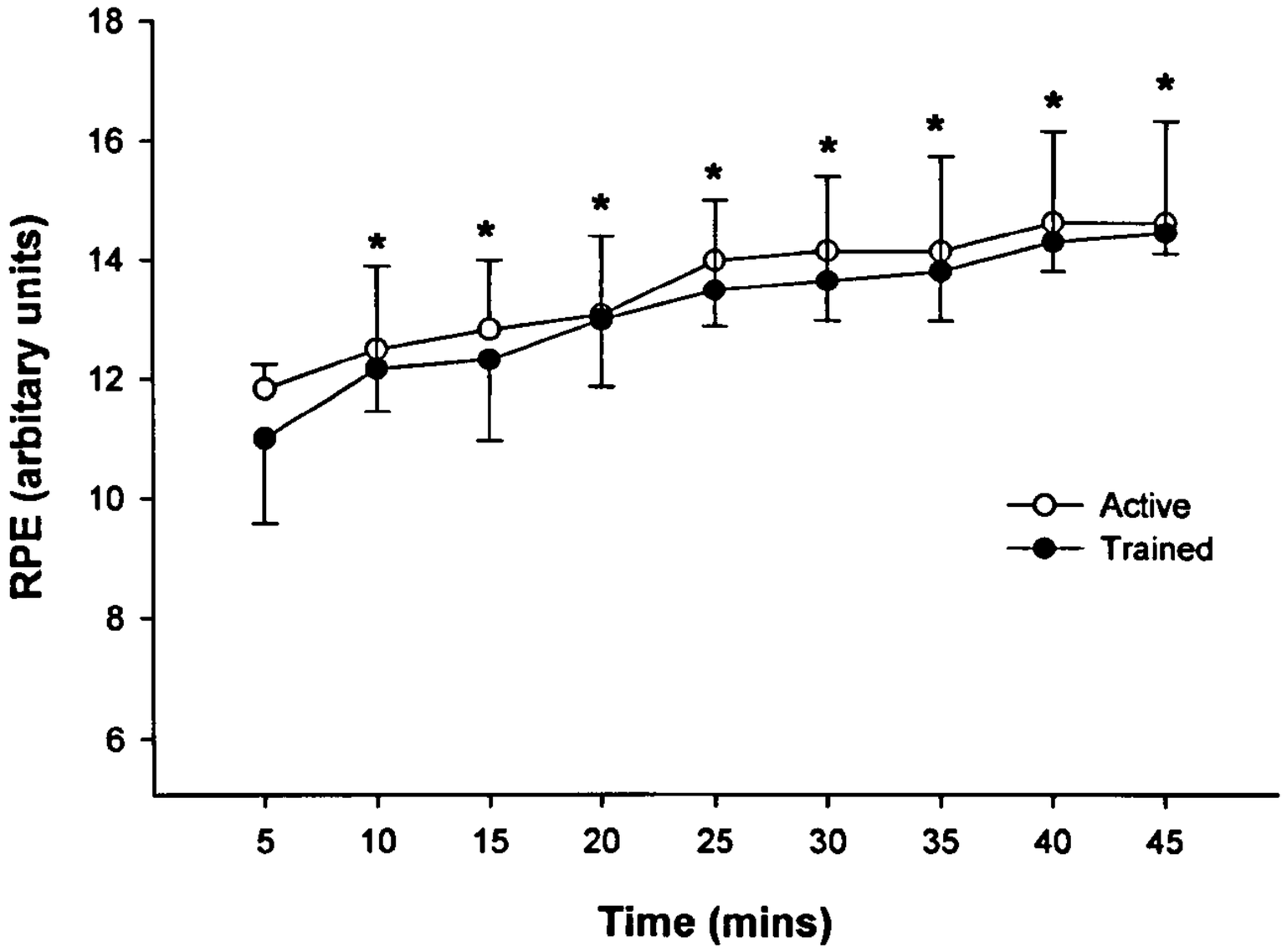


Figure 4.2.4 – RPE of the active and trained subjects during the exercise protocol. * denotes significant difference from pre-exercise values, P<0.05.

4.2.3.3 Muscle function and voluntary activation. Maximal isometric quadriceps muscle force and voluntary activation of the active and trained subjects are displayed in Figure 4.2.5. There was no significant change in maximal muscle force following the exercise protocol in either group (P>0.05). In accordance with MVC force, voluntary activation also showed no significant change following exercise (P>0.05). Individual CVs for MVC force during the 7 days ranged from 2.1 – 4.63 and 1.99 – 5.52% for the active and trained groups, respectively. Similarly, individual CVs for voluntary activation ranged from 1.86 – 3.76 and 1.66 – 2.91% for active and trained groups respectively. These CV values for MVC force and voluntary activation across trials were all within those ranges (2.98 – 6.52 and 1.62 – 5.27%, respectively) outlined in Study 1. Individual differences in MVC force and voluntary activation across trials were also within the 95% repeatability coefficients (± 76.03 N and ± 4.42% respectively) outlined in Study 1.

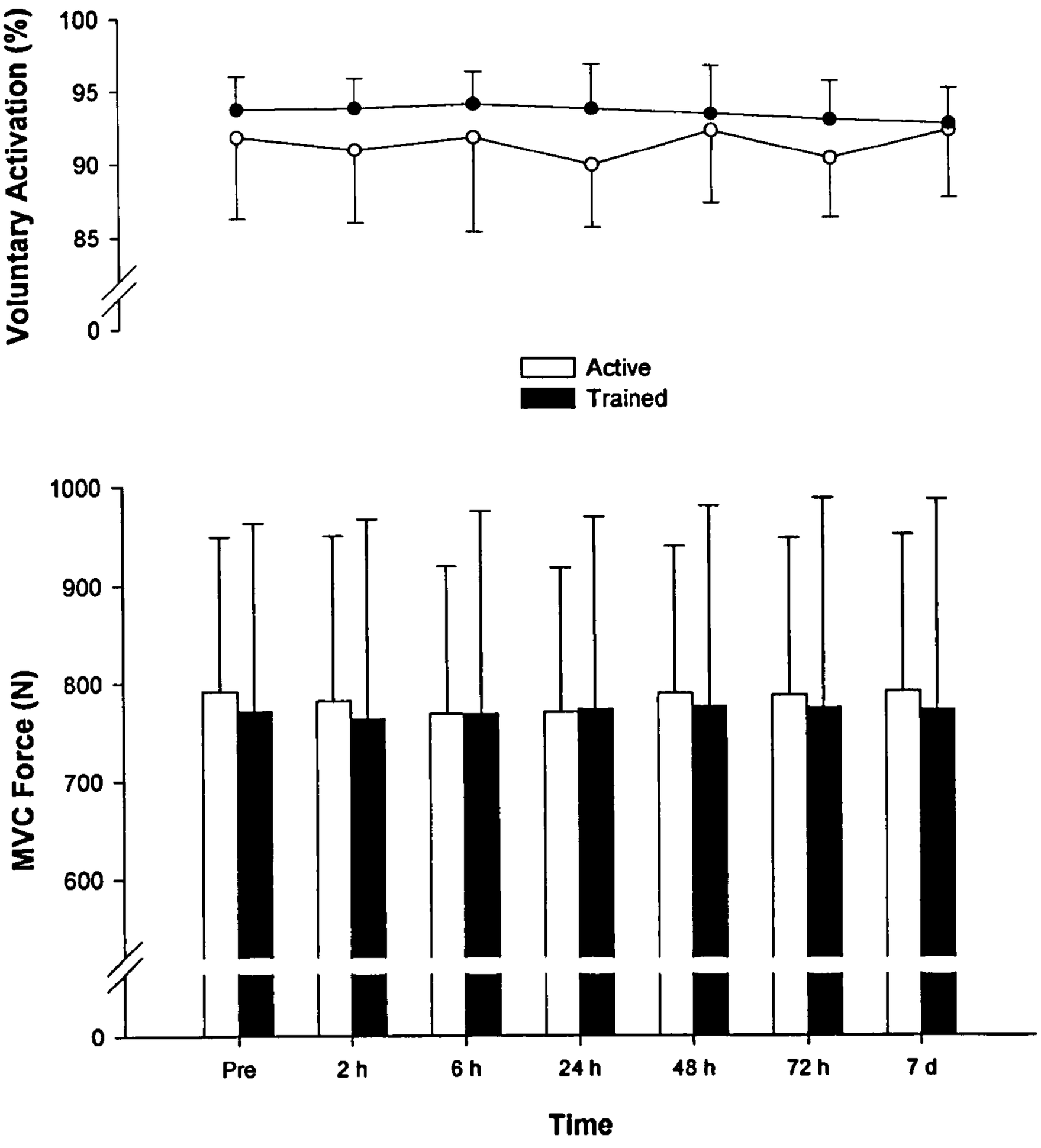


Figure 4.2.5 – MVC force and voluntary activation of the active and trained subjects pre- and post-exercise.

4.2.3.4 Ratings of perceived muscle soreness. Whole body soreness of the active and trained subjects is displayed in Figure 4.2.6. Statistically, there was a significant increase in ratings of perceived whole body soreness in both groups as a result of the exercise bout ($P<0.05$). There was no significant difference in ratings of soreness between groups at any time point post-exercise ($P>0.05$). Ratings of quadriceps soreness (i.e. VAS score) of the active and trained subjects are displayed in Figure

4.2.7. Statistically, there was a significant increase in quadriceps soreness in both groups following exercise ($P<0.05$). There was no significant difference in quadriceps soreness between groups at any time point following exercise ($P>0.05$).

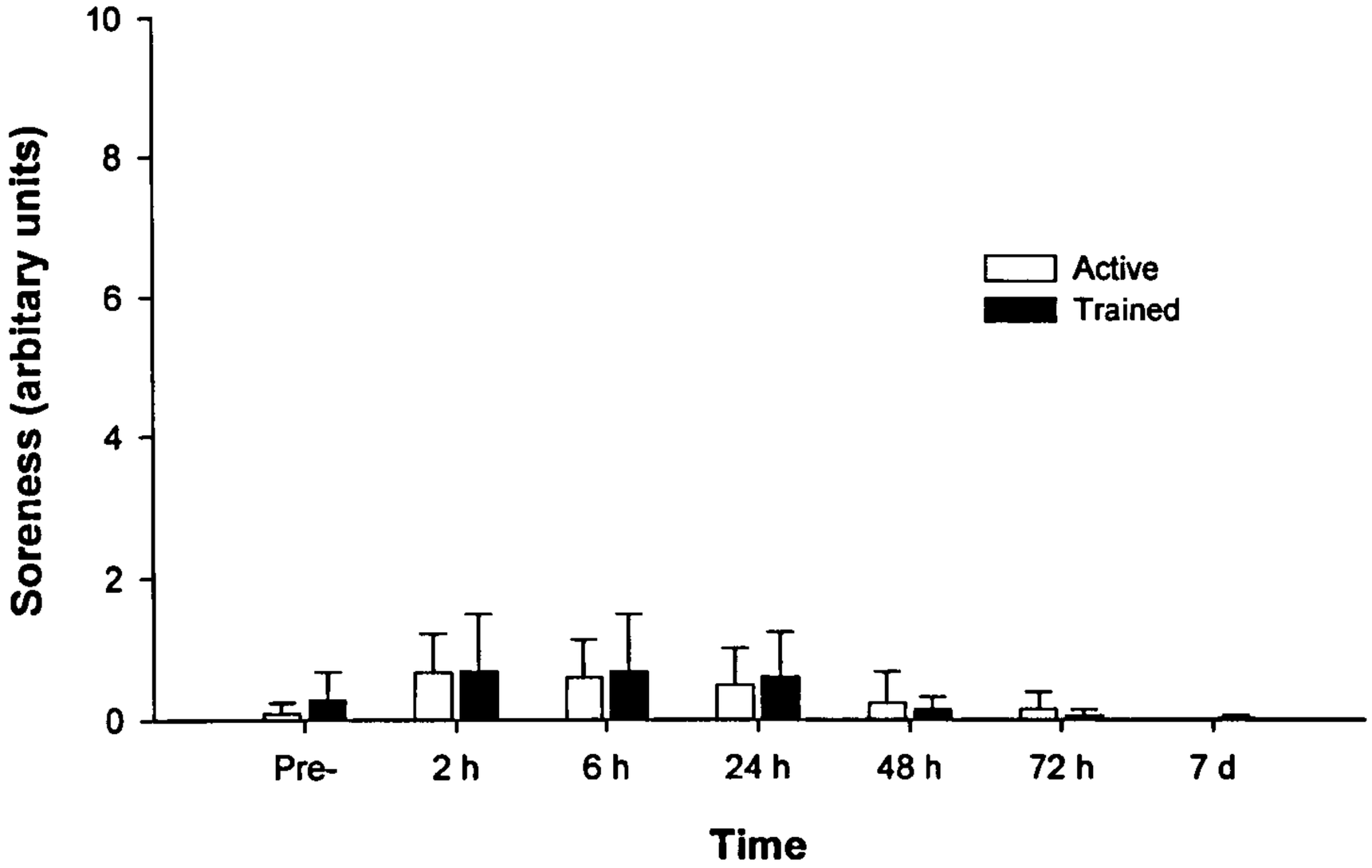


Figure 4.2.6 – Ratings of perceived whole body soreness of the active and trained subjects pre- and post-exercise.

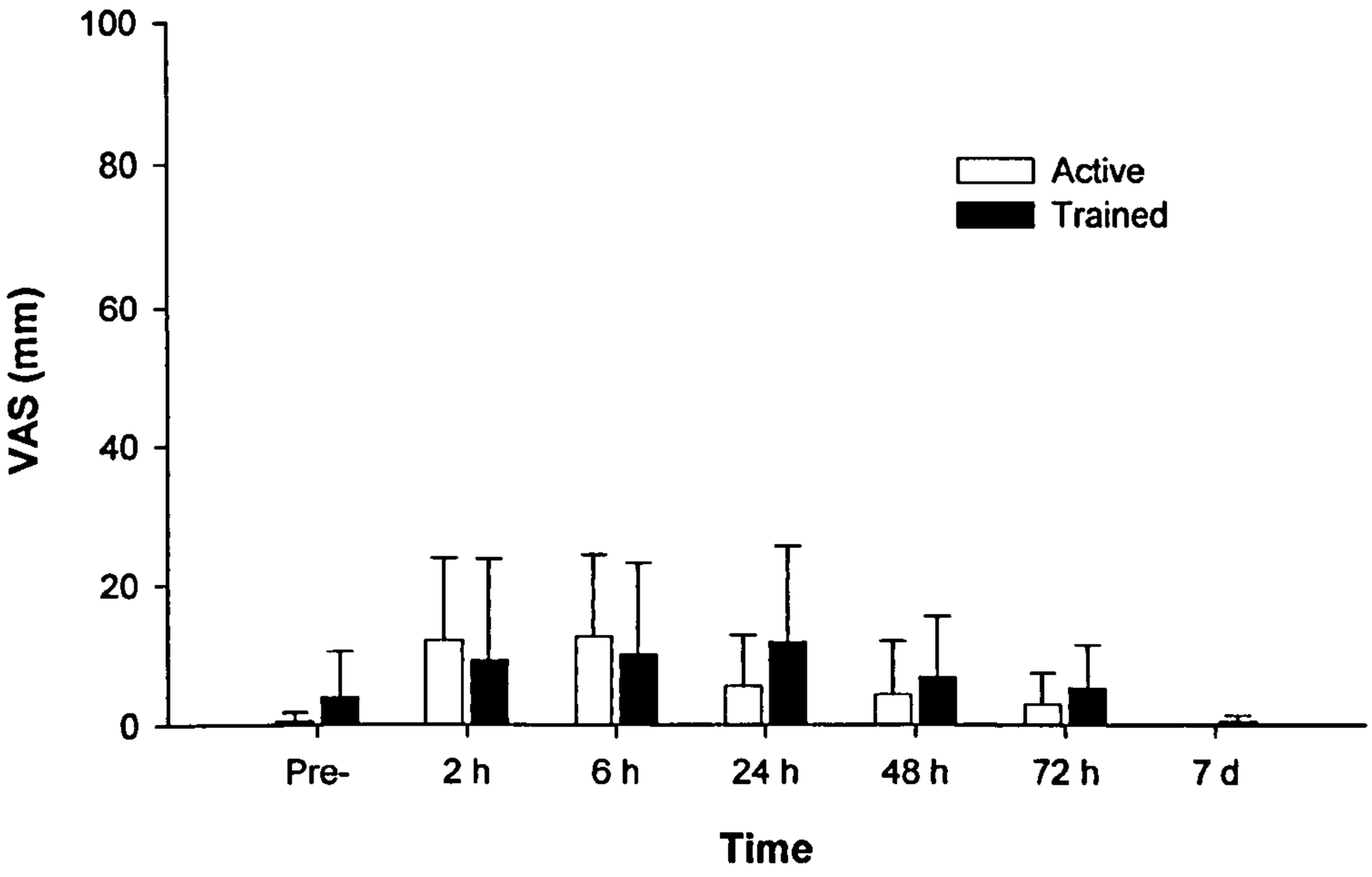


Figure 4.2.7 – Ratings of perceived quadriceps soreness of the active and untrained subjects pre- and post-exercise.

4.2.3.5 Creatine kinase activity. Pre-exercise CK activity of the active and trained groups was 54.1 ± 3.8 and 68.7 ± 35.7 $IU.L^{-1}$ respectively (Figure 4.2.8). Statistically, there was a significant increase in serum CK activity following exercise which peaked at 24 h in both groups ($P<0.05$). Peak CK levels in the active and trained groups were 75.8 ± 22.9 and 96.3 ± 16.4 $IU.L^{-1}$ respectively. There was no significant difference in CK activity between active and trained subjects at any time point ($P>0.05$).

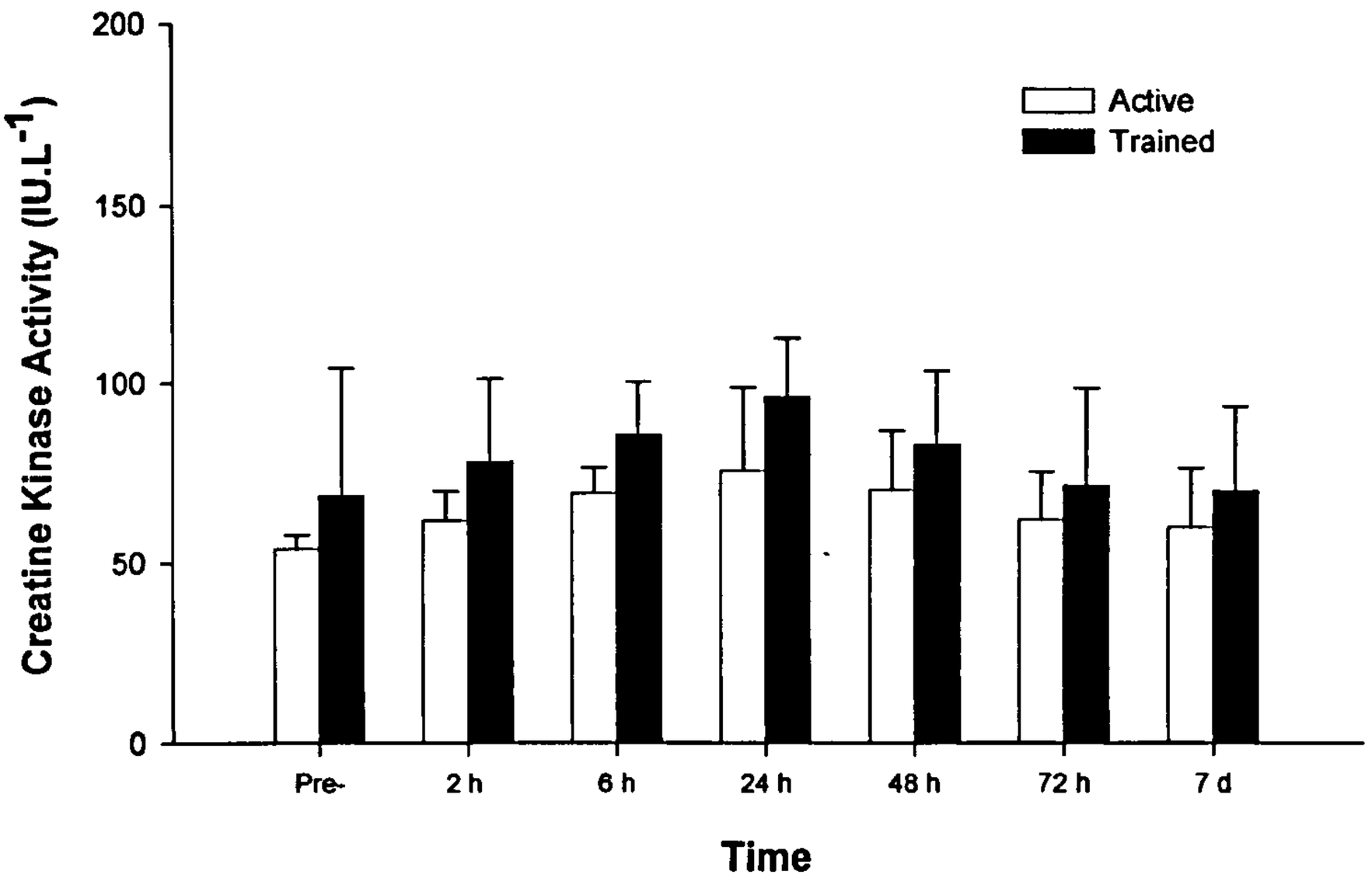


Figure 4.2.8 – Serum CK activity of the active and trained subjects pre- and post-exercise.

4.2.3.6 Muscle HSP content following exercise. HSP70 content for subject 1 (active) and subject 2 (trained) increased to 145 and 124% of pre-exercise levels, respectively (Figure 4.2.9). HSP60 content of subject 1 and 2 increased to 122 and 142% of pre-exercise levels, respectively (Figure 4.2.10).

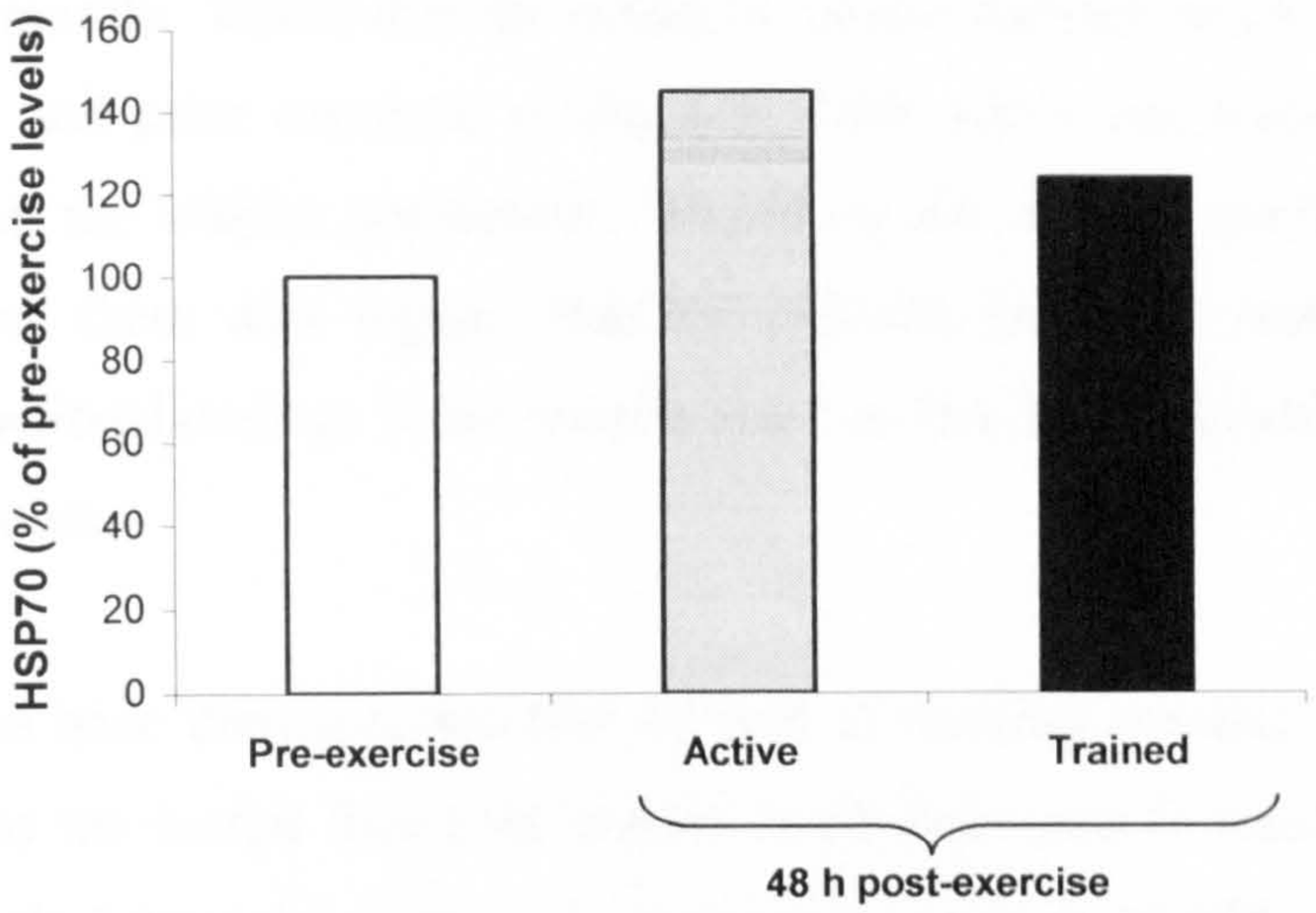


Figure 4.2.9 – HSP70 content of the vastus lateralis for each subject pre- and post-exercise.

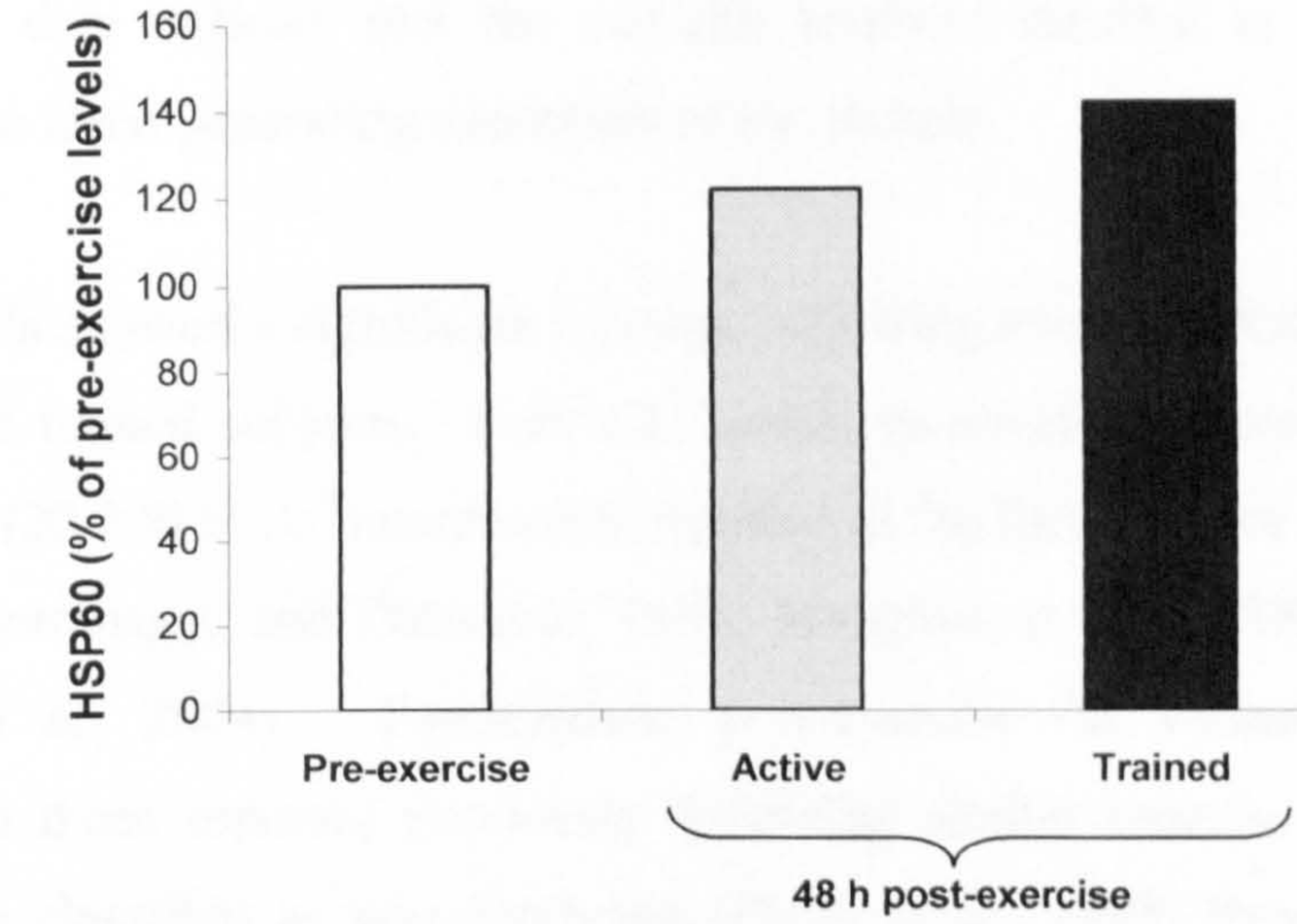


Figure 4.2.10 – HSP60 content of the vastus lateralis for each subject pre- and post-exercise.

4.2.4 DISCUSSION

The aim of the present study was to develop a non-damaging running exercise protocol to utilise as a stimulus to initiate the exercise-induced expression of HSPs in human skeletal muscle. Given that the extent of muscle damage can be influenced by physical fitness and prior exposure to the task, both active and trained individuals were included in the subject population. Based on the indirect markers of muscle damage assessed, these data suggest that the exercise protocol results in no overt structural or functional damage to the muscle and can therefore be considered as 'non-damaging' in nature.

The present data have demonstrated that 45 min of running exercise at an intensity corresponding to the lactate threshold results in no decreases in maximal isometric quadriceps muscle force where voluntary activation levels of 85-95% were observed. These levels of voluntary activation are in agreement with those commonly reported in the literature for attempted maximal contractions of the quadriceps (Shield and Zhou, 2004) and are therefore considered representative of valid maximal efforts. The variations in MVC force observed in the present study (individual CVs ranging from 1.99 to 5.21%) was consistent with that previously observed in Study 1. Taken together, these data indicate that the exercise protocol resulted in no significant reductions in the force generating capability of the muscle.

Serum CK levels showed a significant increase following exercise, peaking at 24 h in both active and trained subjects. Peak CK levels, however, remained within those baseline levels (20-200 $IU.L^{-1}$) commonly reported in the literature for similar subject populations (Hortobagyi and Denaham, 1989; Maughan *et al.*, 1989; Byrne *et al.*, 2001; Close *et al.*, 2004). Furthermore, post-exercise CK values were also in agreement with those reported previously following similar exercise protocols that have also been classified as non-damaging (Pizza *et al.*, 1995; Pyne *et al.*, 1997; Simpson *et al.*, 2006). The observed rise in CK levels therefore appears clinically insignificant in comparison to those levels typically observed following damaging interventions where peak values of 400 – 1000 $IU.L^{-1}$ have been reported (Close *et al.*, 2004; Maughan *et al.*, 1989; Pizza *et al.*, 1995; Simpson *et al.*, 2006). The small

but clinically insignificant increase in circulating CK levels therefore suggests that the exercise protocol resulted in no overt structural damage to the skeletal muscle myocytes. Rather then being representative of damage, such small increases in circulating CK levels may be a result of membrane extrusion of cytosolic components in an attempt to maintain cell viability by helping to reduce oedema (Maglara *et al.*, 2003; Vasilaki *et al.*, 2006).

Both active and trained subjects demonstrated statistically significant increases in ratings of perceived whole body and quadriceps soreness. These values, however, are again of clinical insignificance in comparison with those soreness values (4-7) typically reported following damaging exercise (Pyne *et al.*, 1997; Close *et al.*, 2004). Furthermore, the present levels of soreness are in agreement with those values observed after similar exercise protocols also described as 'non-damaging' (Pyne *et al.*, 1997; Close *et al.*, 2004). The levels of soreness reported here therefore appear to simply reflect the psycho-physiological sensation that exercise has been undertaken rather than genuine symptoms of contraction-induced damage.

The exercise protocol resulted in moderate increases in HSP60 and HSP70 expression at 48 h post-exercise. These elevations of HSP content occurred in the absence of overt structural or functional damage and therefore agree with previous studies (Khassaf *et al.*, 2001, 2003) in that signals arising during exercise other than overt damage to the muscle proteins are capable of initiating a stress response in the skeletal muscle of humans. These signals include (but may not be limited to) exercise-induced increases in muscle temperature, oxidative stress, energy depletion, hypoxia and cytokine production. A more comprehensive investigation as to the signal (s) responsible for the exercise-induced expression of HSPs in response to the non-damaging protocol utilised here will follow in Study 4.

Interestingly, the subject who displayed the lowest increase in HSP70 expression was the subject who was of a trained state. This finding suggests that this subject may not have needed to exhibit a stress response following exercise because the muscle was already pre-conditioned to similar exercise stresses. There appeared to be no differences between subjects in basal expression of HSP70 (as assessed by examination of western blot data). In contrast, however, the trained subject

demonstrated a higher increase in HSP60 expression following exercise. Given that HSP60 is predominantly mitochondrial in location, this elevation may therefore be linked to the higher absolute levels of O_2 flux through the mitochondria demonstrated by this subject during exercise (approximately 15 ml.kg.min^{-1} higher than the active subject). Also of interest was that this subject displayed an approximate 2 fold increase in basal HSP60 content in relation to the active subject (as assessed by examination of western blot data). This finding may be representative of an adaptation to training at the basal level of HSP expression and would appear to agree with findings from animal studies demonstrating that constitutive expression of HSP60 is increased following chronic stimulation (Ornatsky *et al.*, 1995) and treadmill training (Samelan, 2000; Mattson *et al.*, 2000). It is difficult to make accurate conclusions regarding the observed HSP expression to exercise because of the low subject sample that performed the biopsy part of the study (n = 2). However, these preliminary data indicate that the exercise protocol is sufficient to induce HSP expression and also suggest that training status may influence both basal concentrations and the magnitude of the exercise-induced HSP expression. A more comprehensive examination of this hypothesis will be provided in Study 5.

In summary, the present data have confirmed that 45 min of steady state running exercise at an intensity corresponding to the lactate threshold results in no overt structural or functional damage to the skeletal muscle of young healthy active or trained male subjects. The exercise protocol also induced moderate increases in both HSP60 and HSP70 content (n = 2). These data therefore indicate that the exercise protocol is essentially non-damaging in nature and is sufficient to induce HSP expression. The exercise protocol will therefore be utilised in further exercise-related studies in this thesis aimed at examining the time-course of the exercise-induced HSP response and the influence of training status on the magnitude of the stress response following exercise.

Chapter 5

Time-course and differential expression of the major HSP families in human skeletal muscle following acute non-damaging treadmill exercise

This study characterised the time-course of response of the major HSP families in human skeletal muscle of a young active male population following an acute bout of non-damaging running exercise. This work has been published in the Journal of Applied Physiology (see Appendix 2). Aspects of this work were also presented at the 11th Annual Congress of the European College of Sport Science, Lausanne, Switzerland, July 2006, where it received a Young Investigator Award (2nd place oral communication, see Appendix 5).

5.1 INTRODUCTION

Given the array of homeostatic perturbations that occur in contracting skeletal muscle and the unique ability of skeletal muscle to adapt to various stresses, it is not surprising that both acute (Hernando and Manso, 1997; Locke *et al.*, 1990; McArdle *et al.*, 2001; Milne and Noble, 2002; Salo *et al.*, 1991; Skidmore *et al.*, 1995) and chronic exercise (Mattson *et al.*, 2000; Naito *et al.*, 2001; Samelan, 2000) consistently induce increases in HSP content in the skeletal muscle of various animal species. The exercise-induced stress response in rodent models is now relatively well defined where a rapid and dramatic response is typically observed several hours post-exercise (McArdle and Jackson, 2002). Time-course approaches have been adopted following both treadmill running (Hernando and Manso, 1997) and electrical stimulation protocols (McArdle *et al.*, 2001) and have typically demonstrated maximal increases in HSP content within 4 - 12 hours following exercise. Skeletal muscle also adapts to increased contractile activity via an up-regulation of the antioxidant defence network (Powers *et al.*, 1999; Jackson, 2005). The adaptation of such defence systems offer a potential mechanism for the increased tolerance to exercise and protection from contraction-induced damage associated with regular exercise training (Powers *et al.*, 1999; Ji *et al.*, 2006).

Data indicate that exercise is also a sufficient stimulus to increase HSP production in human skeletal muscle. An increase in HSP70 content has been observed in the vastus lateralis following acute 1-legged cycling exercise (Khassaf *et al.*, 2001, 2003), exhaustive knee extensor exercise (Febbraio *et al.*, 2002b) and in the biceps brachii following damaging lengthening contractions (Thompson *et al.*, 2001, 2002, 2003). An increased content of the small HSPs (HSP27 and αB-crystallin) has also been observed in the vastus lateralis following downhill running (Feasson *et al.*, 2002). Despite these initial descriptions, data from human studies are sparse and the exercise-induced stress response of human skeletal muscle remains poorly characterised and understood. Interpretation of data from human studies is often limited to the response of one particular HSP family (most notably HSP70) and is complicated by the variations in timing of tissue sampling between studies, differing subject characteristics (e.g. age,

training status, recent activity levels, gender, nutritional status) and perhaps more importantly, the disparate exercise protocols utilised by investigators. (mode/intensity/duration/damaging/non-damaging). The advantages of using non-damaging exercise protocols to study the exercise-induced stress response of human skeletal muscle have been discussed in Study 2.

Surprisingly, only two studies thus far have investigated the heat shock response of human skeletal muscle following running exercise protocols (Puntschart *et al.*, 1996; Walsh *et al.*, 2001). These authors failed to detect an increase in HSP70 content at 3 h (Puntschart *et al.*, 1996) and 24 h (Walsh *et al.*, 2001) following a period of running at the 'anaerobic threshold' and 70% of $\dot{V}O_{2max}$, respectively. It may be that the absence of a stress response in these studies is due to an insufficient exercise intensity required to mediate an increase in HSP content. However, the above protocols were sufficient to induce an increase in HSP70 gene expression suggesting that the appropriate signalling pathway had been activated. It is therefore likely that the timing of biopsy sampling in the previous studies was not appropriate to allow translation of HSPs to occur. Indeed, Khassaf *et al.* (2001) demonstrated that biopsy samples beyond 24 h post-exercise are needed in order to detect exercise-induced changes in muscle HSP levels following cycling exercise. A comprehensive time-course study of the stress response following running exercise has, however, yet to be performed.

The aim of the present study was to therefore characterise the time-course and pattern of response of the major HSP families in human skeletal muscle following an acute bout of non-damaging treadmill running exercise (developed from Study 2). These include the HSP70 family (HSP70 and HSC70), mitochondrial HSP60 and two members of the small HSP family (HSP27 and αB-crystallin). The response of important antioxidant enzymes of superoxide dismutase and catalase following exercise were also examined. Given recent evidence that baseline HSP levels display marked individual variation (Khassaf *et al.*, 2001), the extent of individual variation of baseline HSPs and antioxidant protein levels was also evaluated.

5.2 METHODS

5.2.1 Subjects. Eight active males volunteered to participate in the study (mean ± SD: age, 24 ± 4 years; weight, 78.9 ± 7.4 kg; height, 1.8 ± 0.05 m; $\dot{V}O_{2max}$, 54.9 ± 4 ml.kg^{-1}.min^{-1}; lactate threshold, 69.8 ± 4.8 % $\dot{V}O_{2max}$). The study was approved by the Ethics Committee of Liverpool John Moores University and all subjects conformed to the criteria outlined in section 3.1.2.

5.2.2 Design. All subjects were initially assessed for $\dot{V}O_{2max}$ (see section 3.3) and lactate threshold (see section 3.4). These tests were separated by at least 48 h. Having then refrained from exercise and prolonged thermal exposure (i.e. baths, saunas, steam rooms, tanning devices etc) for 3-5 days following the lactate threshold test, subjects completed the 45 min non-damaging treadmill running protocol developed in Study 2, at a speed corresponding to their lactate threshold on a motorised driven treadmill (see section 3.8). Muscle biopsies were obtained from the vastus lateralis (see section 3.10) immediately prior to the exercise protocol and at 24 h, 48 h, 72 h and 7 days post-exercise. Muscle samples were analysed for HSP70, HSC70, HSP60, HSP27, αB-crystallin and MnSOD protein content as well as activity of total SOD and catalase (see section 3.11-3.12).

5.2.3 Statistical analyses. Changes in exercise related variables during the exercise protocol and changes in muscle HSP content and antioxidant enzyme activity following exercise were analysed using repeated measurements General Linear Models. Where there was a significant main effect for time, paired *t*- tests with Bonferroni corrections were used for post-hoc analysis. Differences in muscle temperature between pre- and post-exercise were assessed using a students *t*-test for paired samples. In a 'summary of statistics' approach (Altman, 1991), students *t*-test for paired samples were also used to examine pre- to peak changes in HSP content. Peak changes were taken as the time-point at which subjects demonstrated their maximal change in muscle HSP content. Correlations between specific baseline protein levels were assessed using Pearson's

correlation coefficient. All data are presented as means ± SD with P values of <0.05 indicating statistical significance.

5.3 RESULTS

5.3.1 Physiological and thermoregulatory responses to the exercise protocol. The exercise protocol was performed at a running speed of 11.7 ± 0.5 $km.h^{-1}$. Heart rate and ratings of perceived exertion and thermal comfort during exercise are displayed in Table 5.1. All of these variables displayed a significant ($P<0.05$) and progressive linear increase during exercise. Oxygen uptake and blood lactate values showed no significant change during exercise. Specifically, $\dot{V}O_2$ corresponded to 68.8 ± 4.8, 70.5 ± 3.8 and 69.5 ± 3.7 % of $\dot{V}O_{2max}$ at 5-10, 20-25 and 35-40 min of exercise, respectively. Blood lactate values were 3.0 ± 0.4, 3.3 ± 0.6 and 3.5 ± 0.7 $mmol.l^{-1}$ after 15, 30 and 45 min of exercise, respectively. Core and muscle temperature changes during the exercise protocol are presented in Figure 5.1. Exercise induced a significant rise ($P<0.05$) in core temperature increasing from 37.5 ± 0.2 at rest to 39.2 ± 0.3 °C immediately post-exercise. Muscle temperature also exhibited a significant increase ($P<0.05$) during exercise increasing from 36.2 ± 0.7 at rest to 40 ± 0.3°C immediately post-exercise.

Table 5.1 – Heart rate and ratings of perceived exertion (RPE) and thermal comfort (TCS) during the exercise protocol. * denotes significant difference from pre-exercise values, $P<0.05$.

	Time (mins)					
	Pre-	10	20	30	40	Post-
Heart Rate ($b.min^{-1}$)	76 ± 6	164 ± 6*	169 ± 6*	174 ± 5*	179 ± 6*	179 ± 6*
RPE	6	12 ± 1*	13 ± 1*	14 ± 1*	16 ± 2*	16 ± 2*
TCS	5 ± 1	6 ± 1*	7 ± 1*	7 ± 1*	8 ± 1*	8 ± 1*

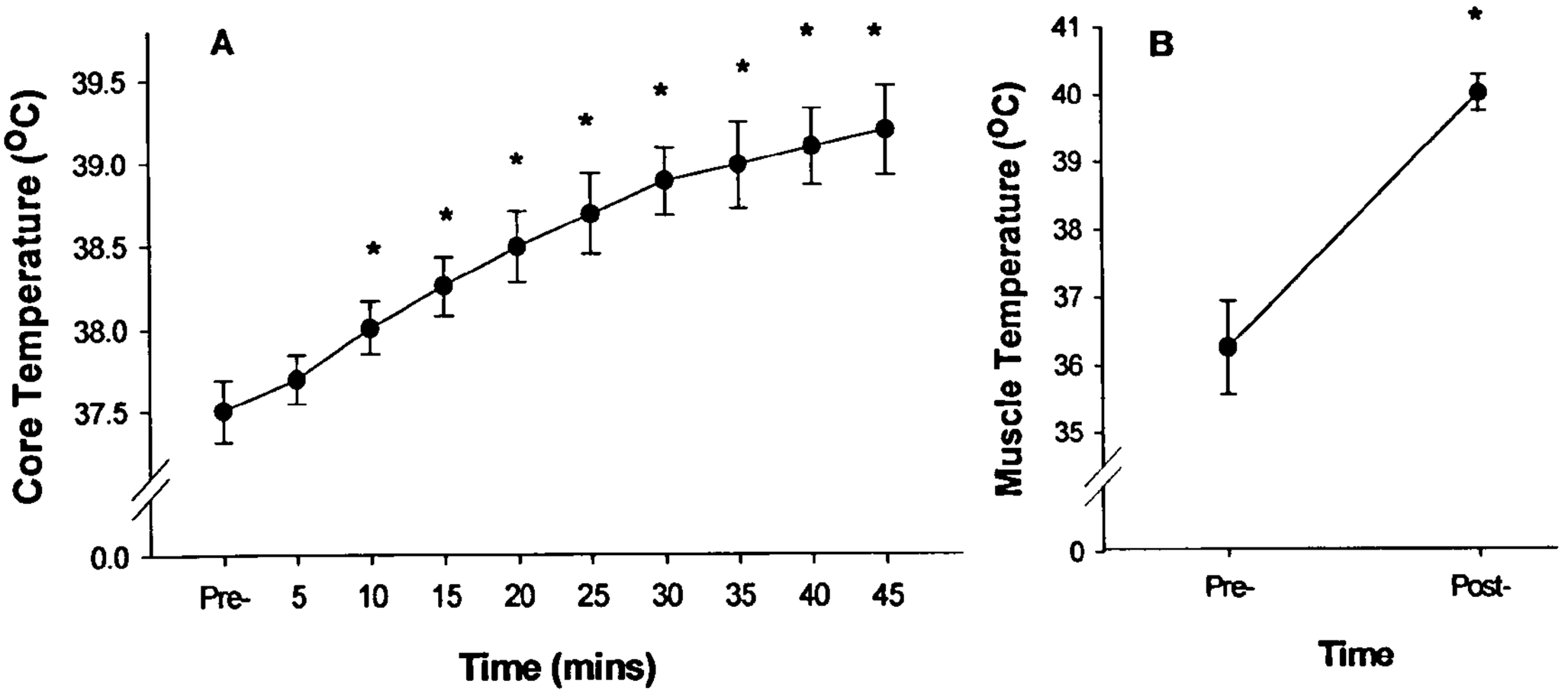


Figure 5.1 – (A) Core temperature during the exercise protocol and (B) muscle temperature of the vastus lateralis immediately pre- and post-exercise. * denotes significant difference from pre-exercise values, $P<0.05$.

5.3.2 Muscle HSP content following exercise. Muscle HSP70 content showed a significant and variable increase following exercise (Figure 5.2 A). This response achieved significance ($P<0.05$) at 48 h (179% of pre-exercise content) and 7 days post-exercise (178% of pre-exercise content). When considering peak responses (Figure 5.2 B) which typically occurred at 48 h post-exercise, HSP70 increased to 210 ± 70% of pre-exercise levels (range, 135 – 366%). The HSP70 response to exercise showed a marked individual variation in both magnitude and time-course of the response. Representative western blots highlighting an example of such individual variation are presented in Figure 5.2 C-F.

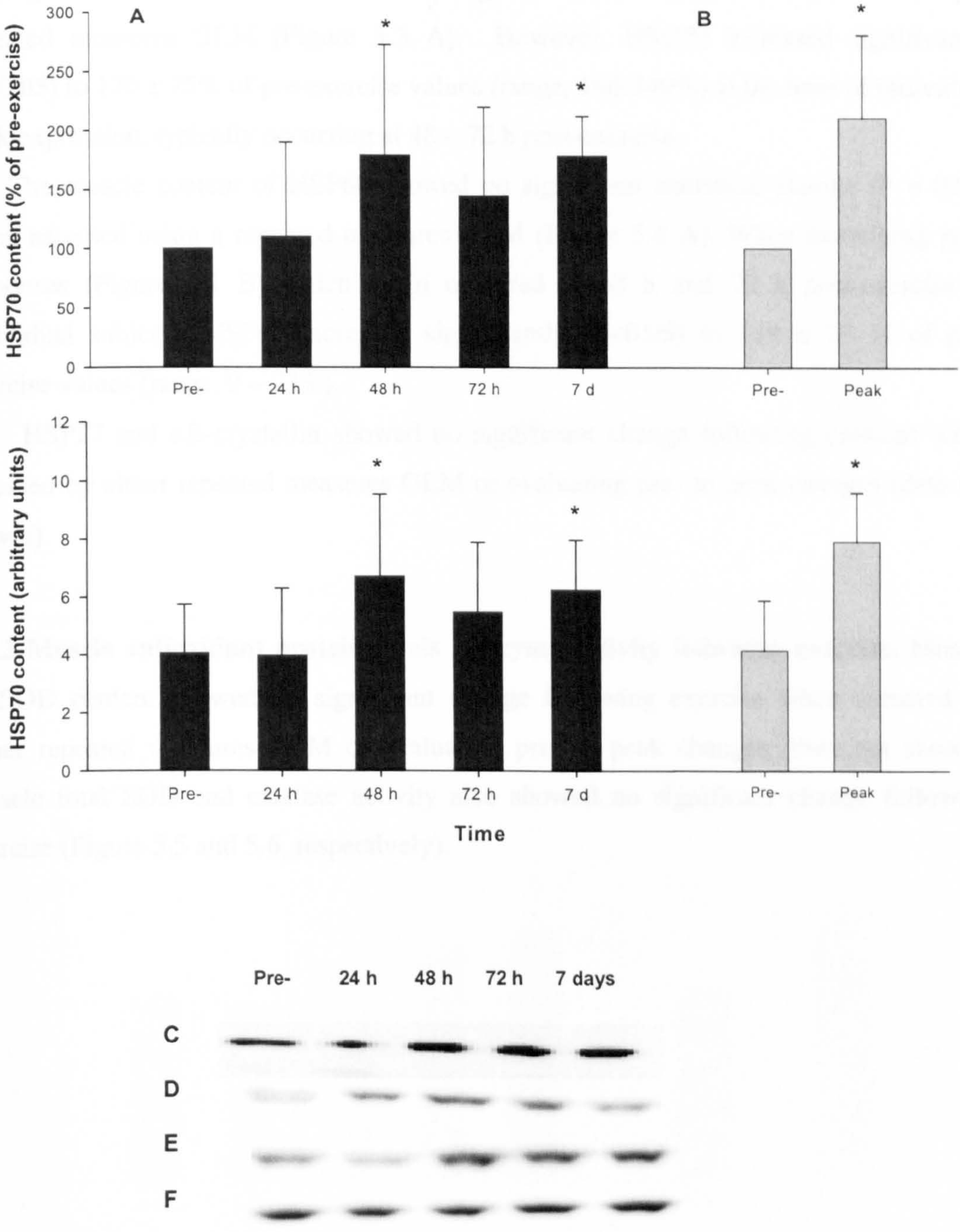


Figure 5.2 – (A) HSP70 content of the vastus lateralis before and after exercise and (B) peak changes in HSP70 content. Upper panel data are expressed as a percentage of pre-exercise levels, lower panel data are presented as absolute arbitrary units. * denotes significant difference from pre-exercise values, $P<0.05$. (C – F) Representative western blots of individual subjects who showed varying HSP70 responses.

The pattern of changes in muscle HSC70 content mirrored the response of HSP70 although this change was not statistically significant (P = 0.08) when assessed using a repeated measures GLM (Figure 5.3 A). However, HSC70 increased significantly (P<0.05) to 170 ± 75% of pre-exercise values (range, 166-340%) at the time of individual peak expression, typically occurring at 48 – 72 h post-exercise.

The muscle content of HSP60 showed no significant statistical change (P = 0.06) when assessed using a repeated measures GLM (Figure 5.4 A). When examining peak responses (Figure 5.4 B) which again occurred at 48 h and 72 h post-exercise in individual subjects, HSP60 increased significantly (P<0.05) to 139 ± 23 % of pre-exercise values (range, 9 – 73%).

HSP27 and αB-crystallin showed no significant change following exercise when assessed by either repeated measures GLM or evaluating pre- to peak changes (data not shown).

5.3.3 Muscle antioxidant protein levels / enzyme activity following exercise. Muscle MnSOD content showed no significant change following exercise when assessed by either repeated measures GLM or evaluating pre- to peak changes (data not shown). Muscle total SOD and catalase activity also showed no significant change following exercise (Figure 5.5 and 5.6, respectively).

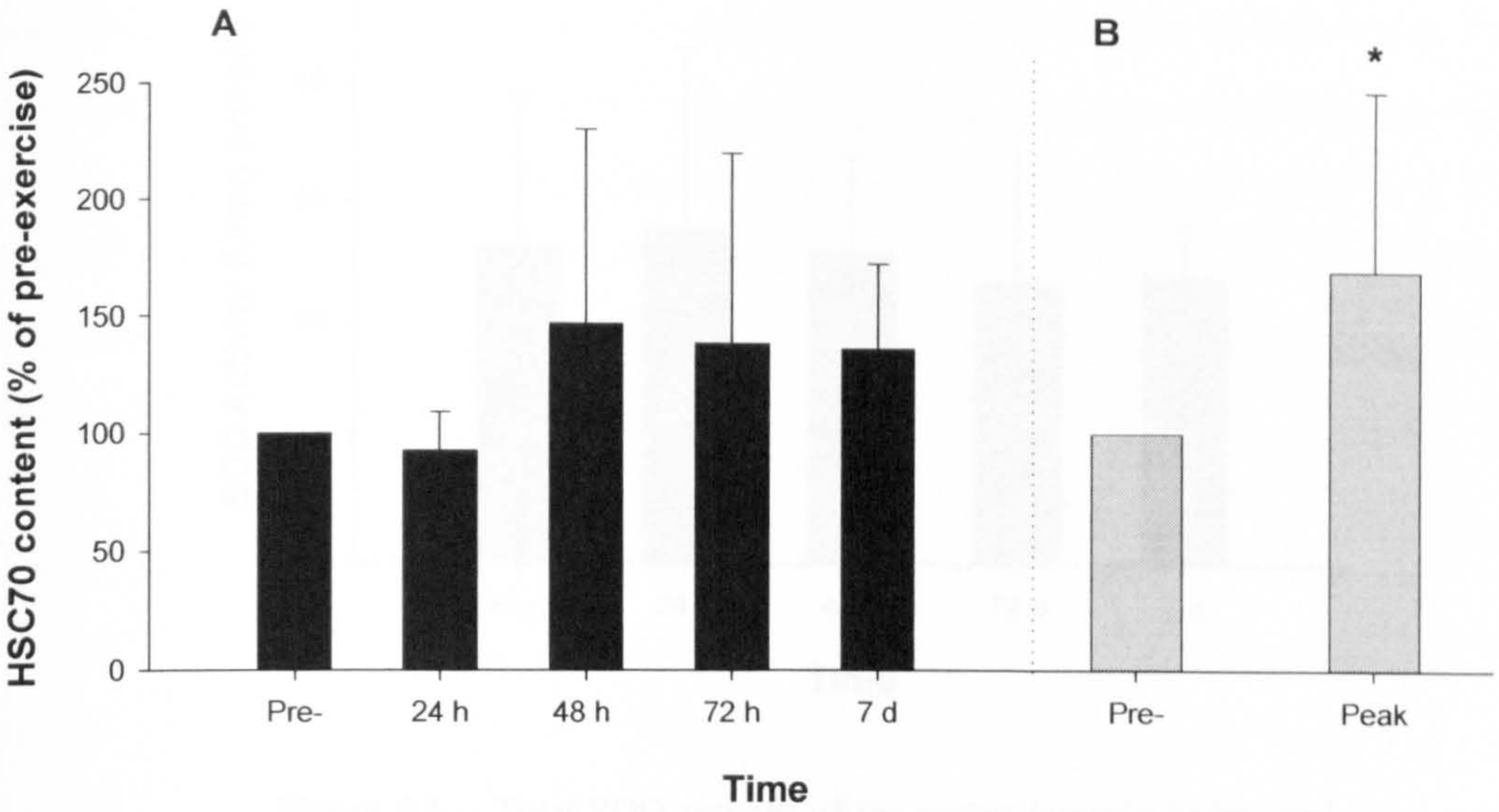


Figure 5.3 – (A) HSC70 content of the vastus lateralis before and after exercise and (B) peak changes in HSC70 content. * denotes significant difference from pre-exercise values, $P<0.05$.

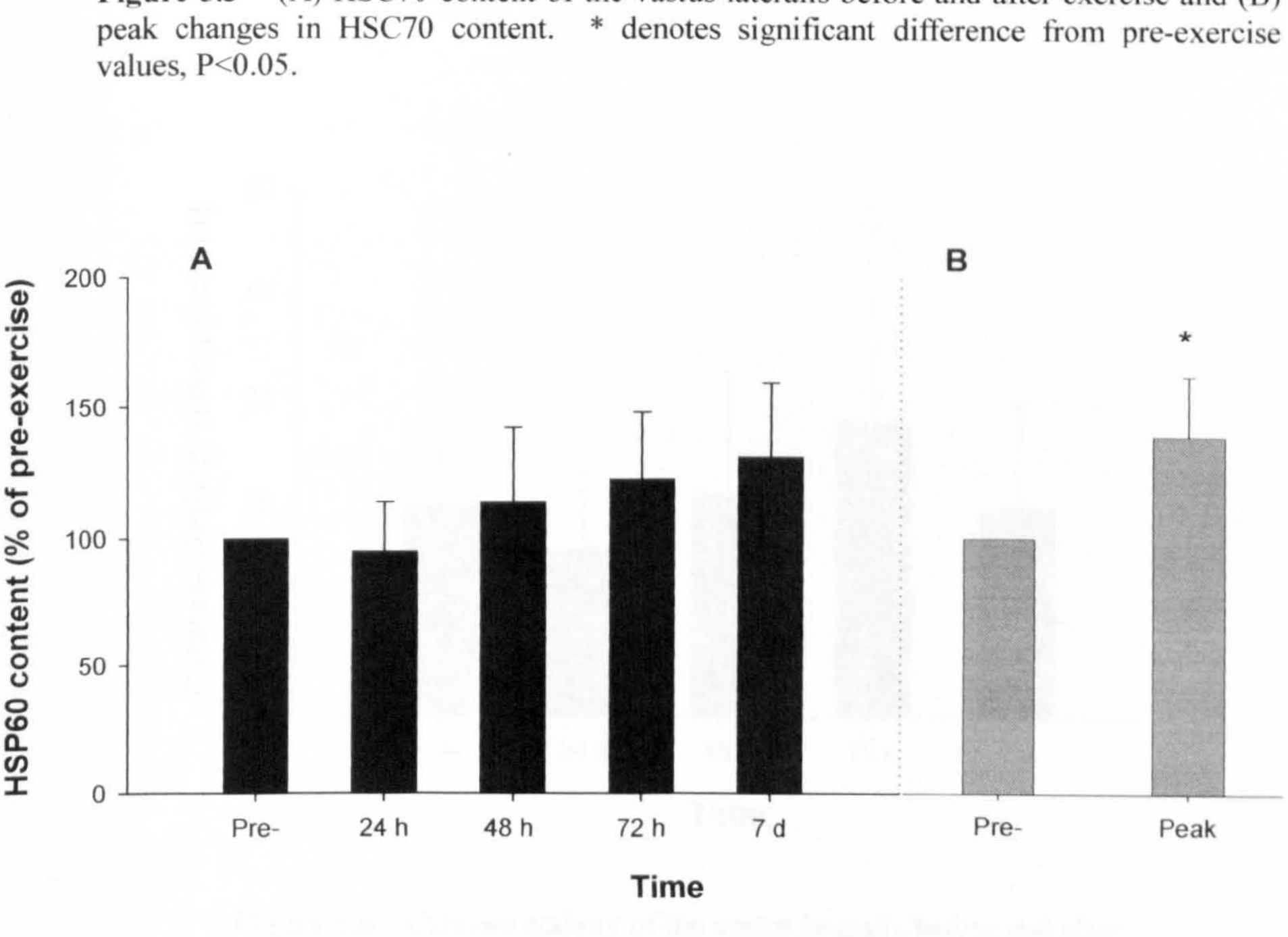


Figure 5.4 – (A) HSP60 content of the vastus lateralis before and after exercise and (B) peak changes in HSP60 content. * denotes significant difference from pre-exercise values, $P<0.05$.

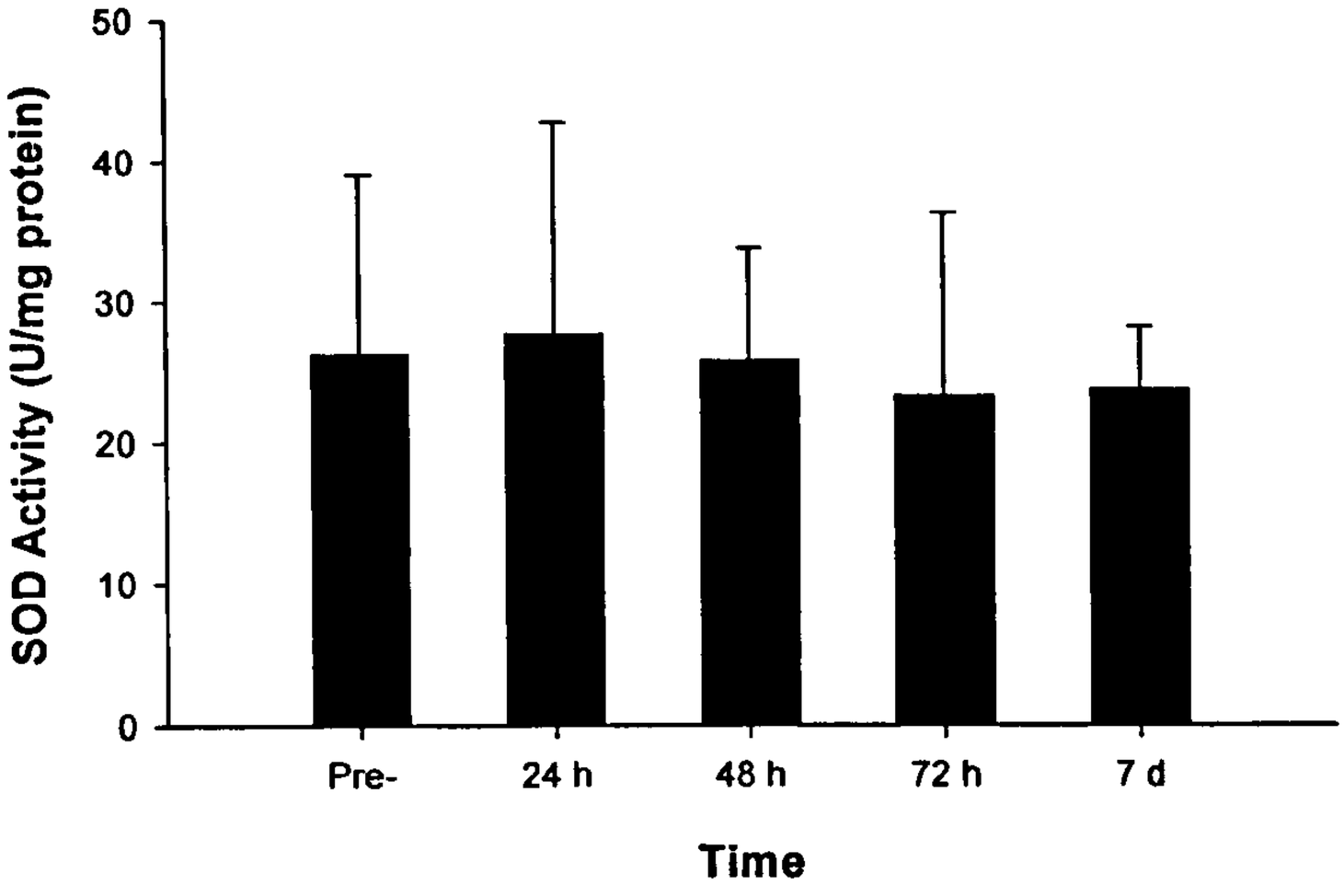


Figure 5.5 – Total SOD activity of the vastus lateralis before and after exercise.

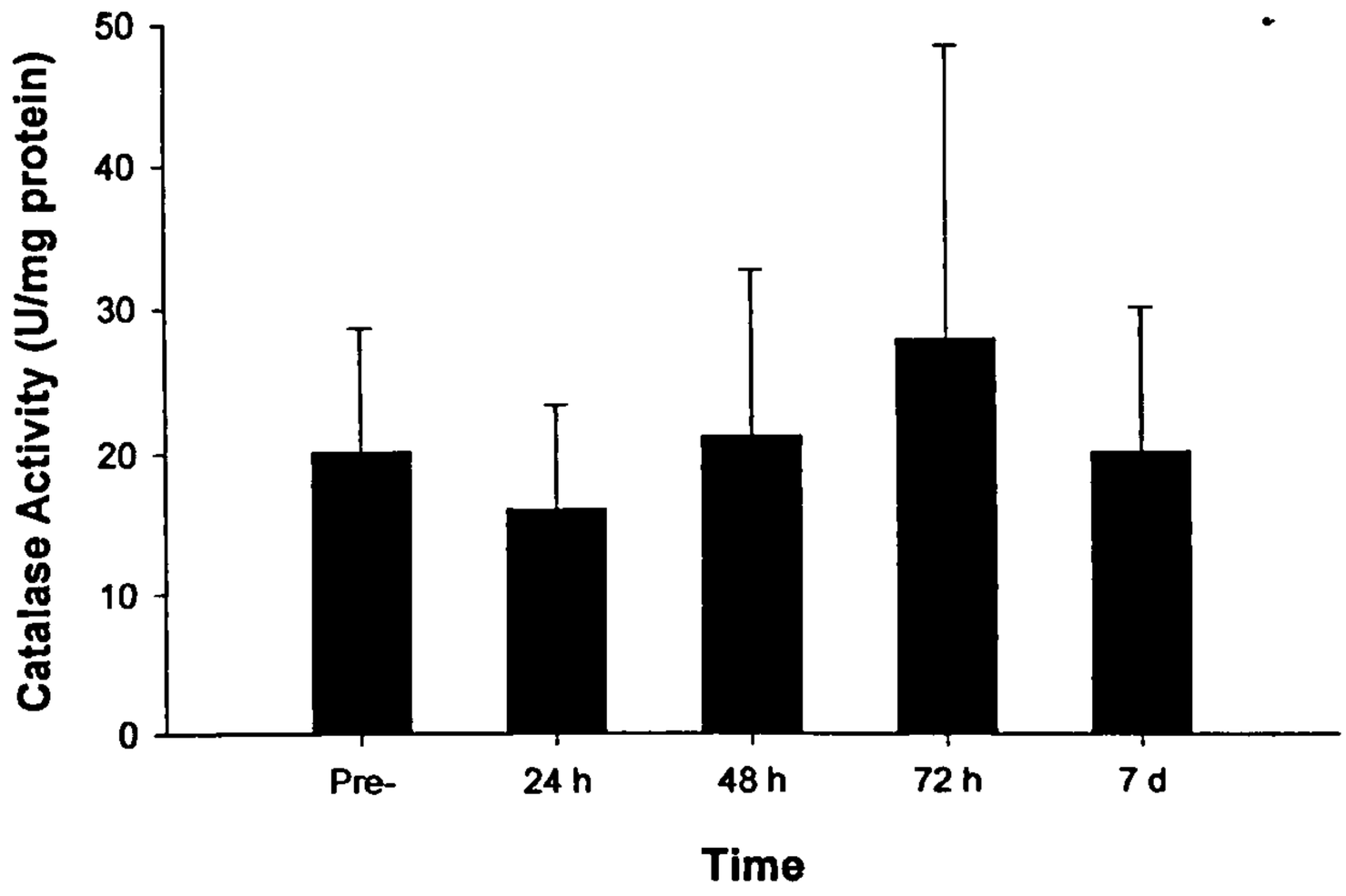


Figure 5.6 – Catalase activity of the vastus lateralis before and after exercise.

5.3.4 Baseline muscle HSPs and MnSOD levels. A comparison of subjects' pre-exercise levels of muscle HSP and MnSOD content are shown in Figure 5.7. Western blots from only 7 subjects are presented due to insufficient tissue available from 1 subject to perform analysis. HSC70, HSP27 and αB-crystallin was constitutively expressed and showed little individual variation between subjects. In contrast, HSP70 and MnSOD expression displayed marked variation exhibiting up to a 3-fold and 1.5 fold difference between subjects, respectively. Of all baseline proteins, correlations were only evident between HSP70 and MnSOD ($r = 0.81$).

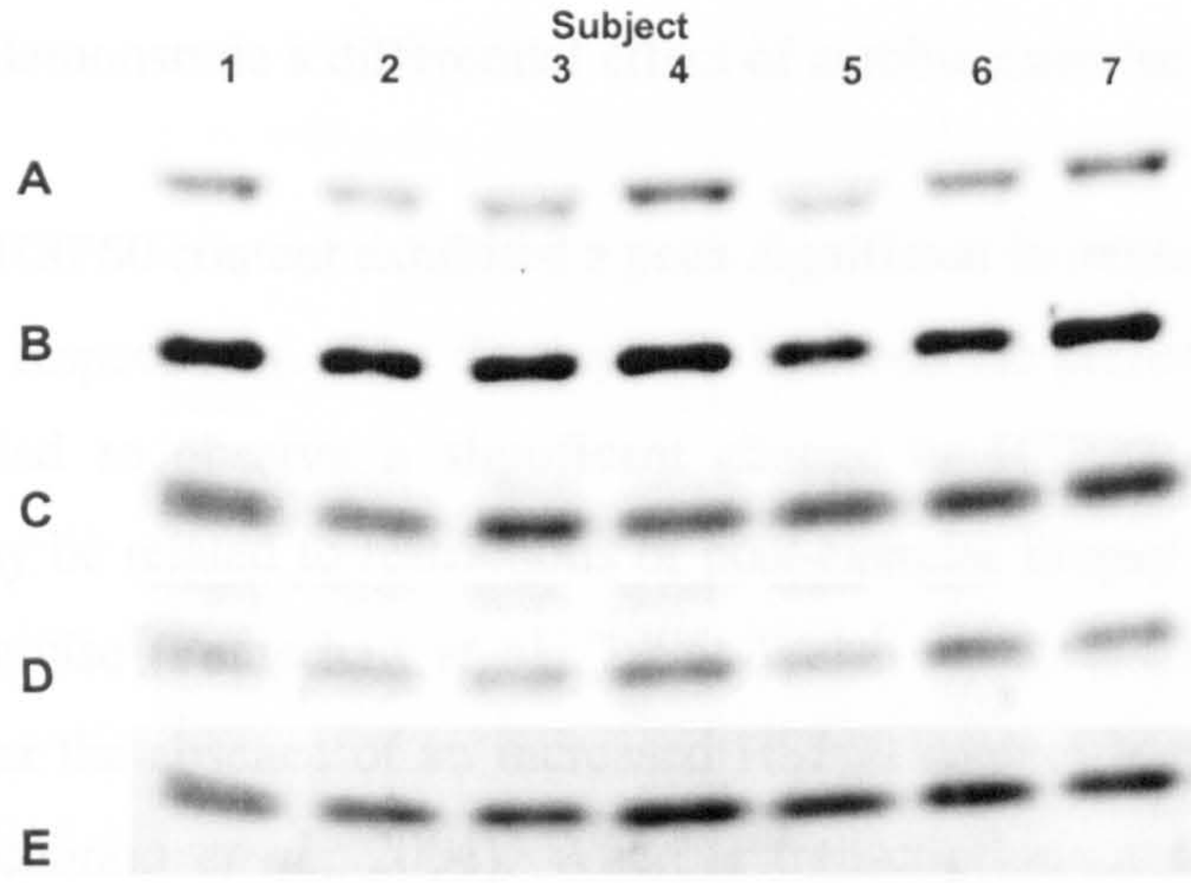


Figure 5.7 – Western blots showing a comparison of subjects' baseline content of (A) HSP70, (B) HSC70, (C) HSP27, (D) MnSOD and (E) αB-crystallin of the vastus lateralis.

5.4 DISCUSSION

The present study has characterised the time-course and magnitude of response of the major HSP families in the skeletal muscle of an active young male population following an acute bout of moderately demanding and non-damaging treadmill exercise. This study provides novel data and has demonstrated that running exercise is a sufficient stimulus to up-regulate the expression of several HSPs (most notably HSP70). Although examination of individual data reveals that 48 h post-exercise appears to be an appropriate time point for which to detect maximal exercise-induced increases in HSP expression, the use of multiple post-exercise biopsy samples is recommended in future studies. This is because the stress response appears highly variable between subjects in that some individuals may not display peak responses to 72 h or 7 days following exercise. Data also demonstrate a differential effect of aerobic exercise on specific HSPs.

Muscle HSP70 and HSP60 content exhibited a peak significant increase of approximately 2-fold and 1.4 fold, respectively. The discrepancy between the present study and those who previously failed to observe a significant change in HSP70 content following running exercise may be related to restrictions of post-exercise biopsy samples to within 1 day following exercise (Puntschart *et al.*, 1996; Walsh *et al.*, 2001). This is also a likely explanation for the absence of an increased HSP60 content immediately post 2 h cycling exercise (Febbraio *et al.*, 2004). Whereas transcription of HSP genes occurs during (Febbraio and Koukoulas, 2000), immediately after (Puntschart *et al.*, 1996) or several hours post-exercise (Walsh *et al.*, 2001), HSP translation appears to require greater than 24 h. It is therefore possible that a stress response may have also been observed in these studies had multiple post-exercise biopsies been performed.

The increase in muscle HSP70 and HSP60 content in the present study is somewhat smaller than that previously observed following 1-legged cycling (Khassaf *et al.*, 2001, 2003). Of note, however, is the difference in training status of the subject groups between studies. Although the present subjects were not specifically trained athletes, they were involved in an average of 2 h physical activity per week (e.g. recreational

sport) and were of higher physical fitness than the sedentary subjects involved in the previous investigations. It may therefore be speculated that sedentary subjects may mount a greater stress response than that of active or trained subjects (whose muscles are somewhat more pre-conditioned to exercise stresses), so as to combat any homeostatic disruption evoked by the exercise stress. This is true in the muscles of rodents whereby the exercise-induced increase in HSP70 in rat soleus muscle is higher in untrained rats compared with endurance trained rats (Smolka *et al.*, 2000). In a similar manner to the HSP70 response, the lower HSP60 response observed here may also be due to the differing characteristics of active and sedentary subjects. For example, the mitochondria of active subjects are likely to be more equipped with other endogenous defence mechanisms to cope with the changes in oxygen flux that occur during exercise. In such cases, a dramatic HSP60 response may therefore not need to be mounted. In line with this is the increased baseline activity of total SOD activity in the present subjects compared with the sedentary subjects studied by Khassaf *et al.* (2001, 2003). An investigation of the influence of training status on the magnitude of the stress response will follow in Study 5.

Although the present data displayed individual variation in both the time-course and magnitude of HSP70 responses (see individual western blots Figure 5.2 C-E), this variation was substantially lower than that previously observed by Khassaf *et al.* (2001). This is perhaps attributable to the more 'tightly controlled' exercise protocol utilised in the present study. When exercising at the lactate threshold (as opposed to % of $\dot{V}O_{2max}$), relatively homogenous physiological (both cardiac and metabolic) responses are observed (Baldwin *et al.*, 2000). This is true even when comparisons are made between trained and untrained subjects (Baldwin *et al.*, 2000). These authors speculated that such homogenous responses may be due to similar fibre type recruitment patterns. The latter appears relevant in an evaluation of the stress response considering data from rodent studies suggesting that HSP70 expression in the skeletal muscle of rodents following treadmill running displays an intensity-dependent relationship that is partly reflective of muscle recruitment patterns (Milne and Noble, 2002).

The small HSPs are thought to play an important role in the remodelling of myofibrillar structure following stressful and damaging insults (Mounier and Arrigo, 2002). It has therefore been suggested that the small HSPs may be particularly active in the recovery process following exercise-induced muscle damage. In agreement with this, an increased expression of HSP27 has been consistently observed at 48 h post lengthening contractions of the biceps (Thompson *et al.*, 2001, 2002, 2003). Feasson *et al.* (2002) also observed an approximate 2-fold increase in both HSP27 and αB-crystallin at 24 h following a 30 min downhill running protocol. In contrast to the above studies, the present data revealed no increases in muscle content of the small HSPs. This may be due, in part, to the non-damaging nature of our exercise protocol which does not appear to cause any overt structural or functional damage in active young male populations. It is possible therefore that whereas the HSP70 and HSP60 proteins may be up-regulated during exercise by oxidative, thermal, metabolic or cytokine signals, the small HSPs are more responsive to contractile-induced mechanical stresses. It may also be that the relatively high baseline levels of both small HSPs were already sufficient to counteract any stresses which the contractile and structural proteins encountered during exercise.

It was not the intent of the present study to provide a comprehensive analysis as to the possible signals initiating the stress response during exercise. Exercise-induced hyperthermia is routinely suggested as a possible factor responsible for inducing an up-regulation of HSPs following exercise (Fehrenbach and Niess, 1999; Lancaster and Febbraio, 2005; Liu and Steinacker, 2001; Liu *et al.*, 2006). Despite this hypothesis, only one study has previously reported on the extent of muscle temperature change with their chosen exercise protocol (Febbraio and Koukoulas, 2000). In the current study, an increase in both muscle (4 °C) and core (2 °C) temperature was observed, demonstrating a local and systemic hyperthermia effect. At present, it is therefore difficult to conclusively dismiss the role of increased muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle. The role of exercise associated hyperthermia in contributing to the exercise-induced production of HSPs will therefore be addressed in Study 4.

Of all baseline protein levels examined in the present study, only HSP70 and MnSOD showed large individual variation. This inter-individual variation in resting muscle HSP70 levels is not novel and has been demonstrated previously (Khassaf *et al.*, 2001). Data from rodent studies have shown that HSP70 is preferentially expressed in muscles with a high proportion of oxidative fibres (Hernando and Manso, 1997; Kelly *et al.*, 1996; Locke *et al.*, 1991; Locke and Tanguay, 1996). It is possible therefore that the variations in baseline HSP70 levels observed here may be due to differing fibre type characteristics between subjects. The author was unable to quantify muscle fibre types, however, due to the small tissue samples obtained by the chosen biopsy technique. Nevertheless, the observation of moderate correlations between HSP70 and MnSOD protein levels suggests that differences in baseline expression of HSP70 between individuals may be related to differences in fibre type content.

In summary, the present data demonstrate that the skeletal muscle of healthy active young male subjects responds to a period of moderately demanding and non-damaging running exercise via a selective up-regulation several HSPs (predominantly HSP70). Given the well documented cytoprotective role of these proteins, it is likely that their increased expression following exercise functions to restore cellular homeostasis, facilitate cellular remodelling and to offer increased protection against further stressful insults. Further studies are required to examine the HSP responses to aerobic exercise in specific subject populations (i.e. training status specific), to determine the precise physiological role of this increased expression and to investigate the possible signals during exercise inducing an up-regulation of HSP expression. The role of elevated muscle and core temperature in contributing to the exercise-induced expression of HSPs will thus be evaluated in Study 4.

Chapter 6

The role of elevated muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle

Having established the time-course of the exercise-induced stress response in Study 3, the work presented in this Chapter aimed to evaluate the role of elevated muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle in a young active male population. This work was presented at the 11th Annual Congress of the European College of Sport Science, Lausanne, Switzerland, July 2006, where it received a Young Investigator Award (2nd place oral communication, see Appendix 5).

6.1 INTRODUCTION

The stress of exercise induces an array of homeostatic perturbations. It is therefore difficult to isolate the precise stressor(s) that is responsible for initiating the exercise-induced stress response. Exercise associated hyperthermia is routinely suggested as a possible signal responsible for inducing an increased production of HSPs following exercise (Fehrenbach and Niess, 1999; Lancaster and Febbraio, 2005; Liu and Steinacker, 2001; Liu *et al.*, 2006). Indeed, heat shock was the first known stressor to induce an increase in cellular HSP content (Ritossa, 1962; Tissieres *et al.*, 1974). Both *in vitro* (Maglara *et al.*, 2003) and *in vivo* (Oishi *et al.*, 2002, 2003) heating protocols have also been shown to induce an increased HSP70 content in C_2C_{12} skeletal muscle myotubes and rodent muscle, respectively. Furthermore, the exercise-induced production of HSP70 in soleus muscle of rodents is enhanced when exercise is performed under elevated ambient temperatures, an effect attributed to significantly higher body temperatures in these conditions (Kim *et al.*, 2004).

In Study 3, a significant increase was observed in HSP70, HSC70 and HSP60 content of the vastus lateralis muscle following a non-damaging running exercise protocol. The exercise protocol also induced significant increases in core (2°C) and muscle (4°C) temperature suggesting that exercise associated hyperthermia may at least, in part, contribute to the exercise-induced production of HSPs. Elevations in muscle temperature *per se* can also induce increases in reactive oxygen species (Salo *et al.*, 1991; Zuo *et al.*, 2000), also thought to be a potent activator of the exercise-induced stress response (see section 2.5.2). However, no data are available that have tested the specific role of increased muscle and/or core temperature as the primary activator of the exercise-induced stress response in human skeletal muscle.

An investigation of this hypothesis using an exercise related stress would prove extremely difficult as muscle and core temperature would have to be increased independent of each other during exercise. This is technically challenging since contracting muscle is the major site of heat production during exercise (Febbraio and Koukoulas, 2000). The use of passive heating protocols, however, provides a controlled methodological approach by which both muscle and core temperature can

be elevated in isolation or in combination with each other. Passive heating therefore provides an avenue by which to elevate muscle and core temperature to a similar extent as that typically observed during exercise, whilst minimising the circulatory, metabolic and hormonal demands of exercise (Starkie *et al.*, 1999; Gregson *et al.*, 2002).

The aim of the present study was to therefore examine the heat shock response of human skeletal muscle to a passive heating protocol of physiological relevance. A novel and specially designed experimental model was employed in which the muscle (vastus lateralis) temperature of one limb was passively heated to a similar temperature to that previously observed during moderately demanding aerobic exercise (Study 3), whilst the temperature of the contra-lateral limb was maintained at baseline levels. This heating protocol was also advantageous at it initiated a similar systemic thermoregulatory response to exercise (Study 3), thus enabling us to also examine the independent influence of local *vs* systemic increases in heat production in contributing to the intra-muscular expression of HSPs. It was hypothesised that an increase in muscle HSP content would occur in the heated limb only, thereby confirming a role of increased muscle temperature *per se* in contributing to the exercise-induced stress response of human skeletal muscle.

6.2 METHODS

6.2.1 Subjects. Seven healthy young active males volunteered to participate in the study (mean ± SD: age, 23 ± 3 years; weight, 77.8 ± 6.7 kg; height, 1.78 ± 0.04 m; $\dot{V}O_{2max}$, 55.2 ± 2.6 ml.kg^{-1}.min^{-1}). The study was approved by the Ethics Committee of Liverpool John Moores University and all subjects conformed to the criteria outlined in section 3.1.2.

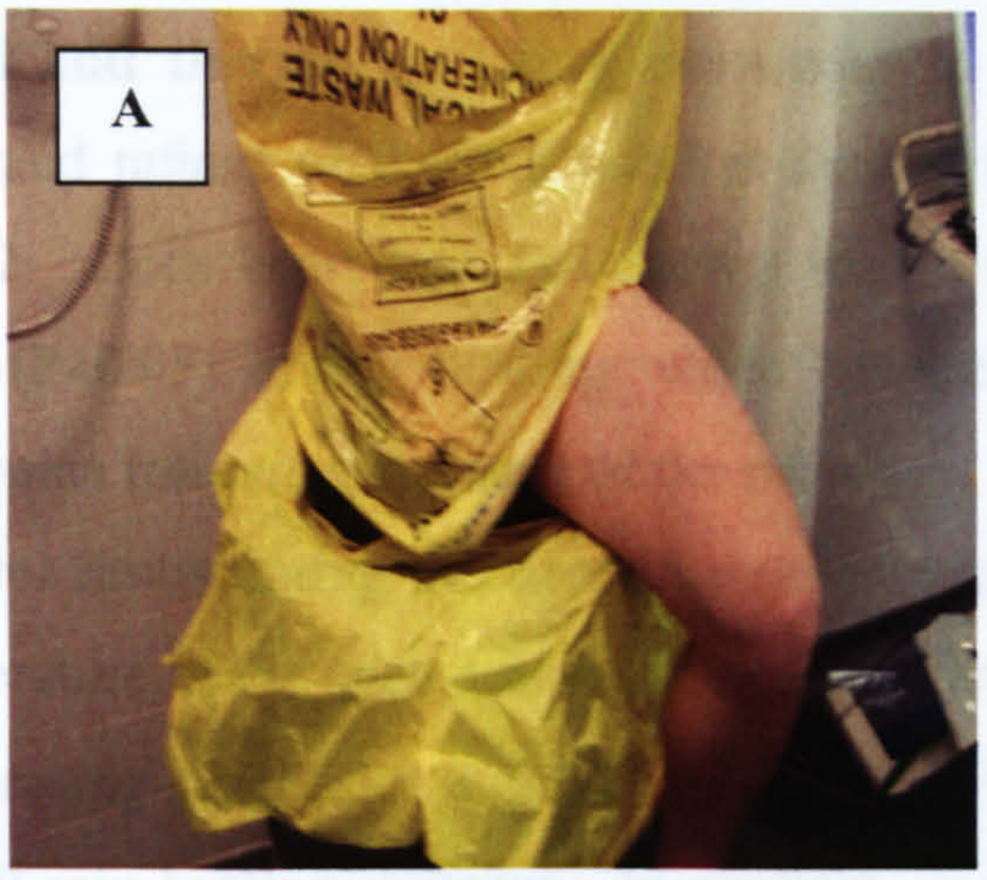
6.2.2 Design. Having refrained from exercise and thermal exposure for at least 4 – 5 days (although brief daily showers were acceptable, subjects refrained from prolonged thermal exposure, e.g. bathing, saunas, tanning devices etc, throughout the study period), subjects underwent a passive heating protocol of 1 h duration in which one leg was immersed in a tank containing warm water. The contra-lateral limb

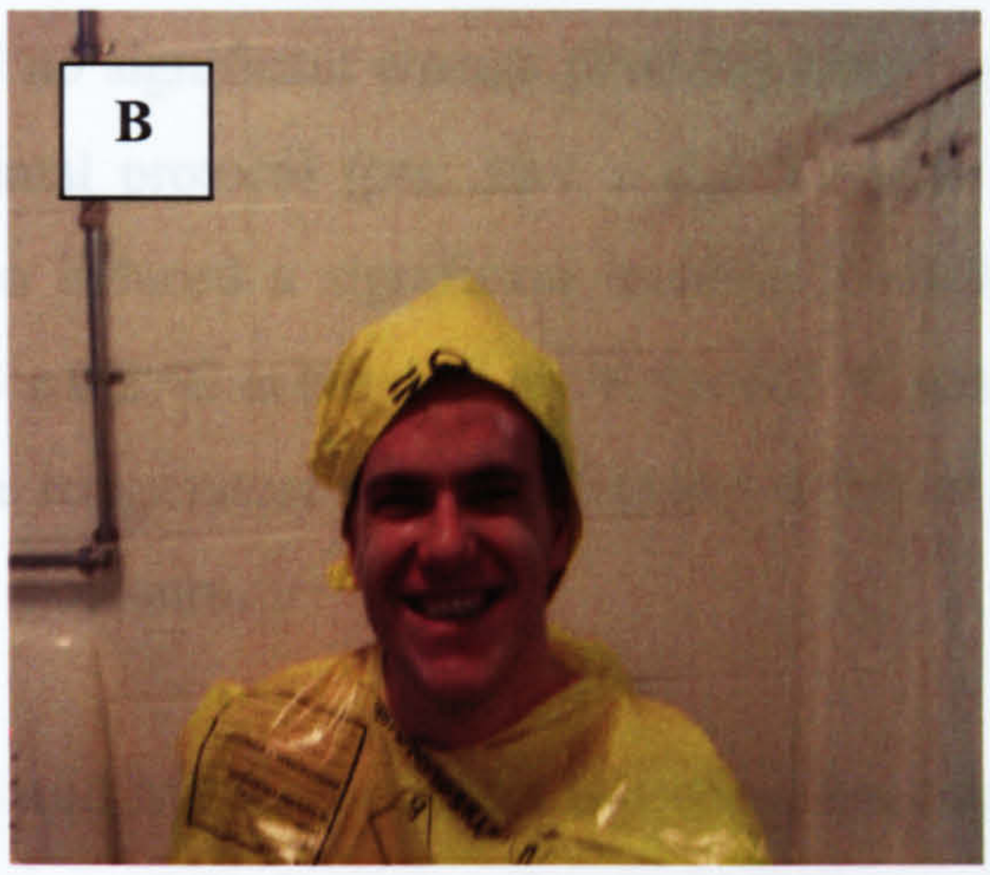
remained outside the tank and did not receive any exposure to heat stress and thus served as a 'control' leg. Muscle biopsies were obtained from the vastus lateralis (see section 3.10) of both legs immediately prior to and at 48 h and 7 days post-heating (these time-points were selected on the basis of the time-course approach in Study 3 examining the stress response following acute non-damaging running exercise in a similar subject population). Muscle samples were analysed for HSP70, HSC70, HSP60, HSP27, αB-crystallin and MnSOD content as well as superoxide dismutase and catalase activity (see section 3.11-3.12).

6.2.3 Heating Protocol. Subjects underwent a passive heating protocol of 1 h duration where they were immersed in warm water (maintained at 45.2 ± 0.8 °C) to the level of the gluteal fold in a specially designed tank. Only one limb was immersed while the contra-lateral leg rested over the side of the water tank. This leg therefore served as a control limb and received no exposure to heat stress. Subjects wore a T-shirt during the protocol and also wrapped their upper torso and head in plastic lining so as to prevent heat loss from the core and head. Heart rate (see section 3.2.1) and ratings of thermal comfort (see section 3.6.2) were recorded at 10 min intervals during heating. Core temperature was measured continuously during the heating protocol (see section 3.7.1) whilst muscle temperature of the vastus lateralis of both limbs was measured immediately pre-, at 20 min intervals throughout and immediately post- the experimental protocol (see section 3.7.2). The ambient temperature of the laboratory during each heating session was approximately 19.1 ± 1.2 °C. Fluid intake was not permitted at any time during heating. An illustration of the experiment set-up is shown in Figure 6.1.

6.2.4 Statistical analyses. Changes in heart rate, ratings of thermal comfort and core temperature during heating were analysed using a one-way repeated measures general linear model (GLM). Changes in muscle HSP content, antioxidant protein content / enzyme activity and muscle temperature (during heating) between legs were analysed using a two-way mixed design GLM where the within factor was time and between factor was condition (heated leg *vs* control leg). Where there was a significant main effect for time, paired *t*- tests with Bonferroni corrections were used for post-hoc

analysis. All data are presented as means ± SD with P values of <0.05 indicating statistical significance.





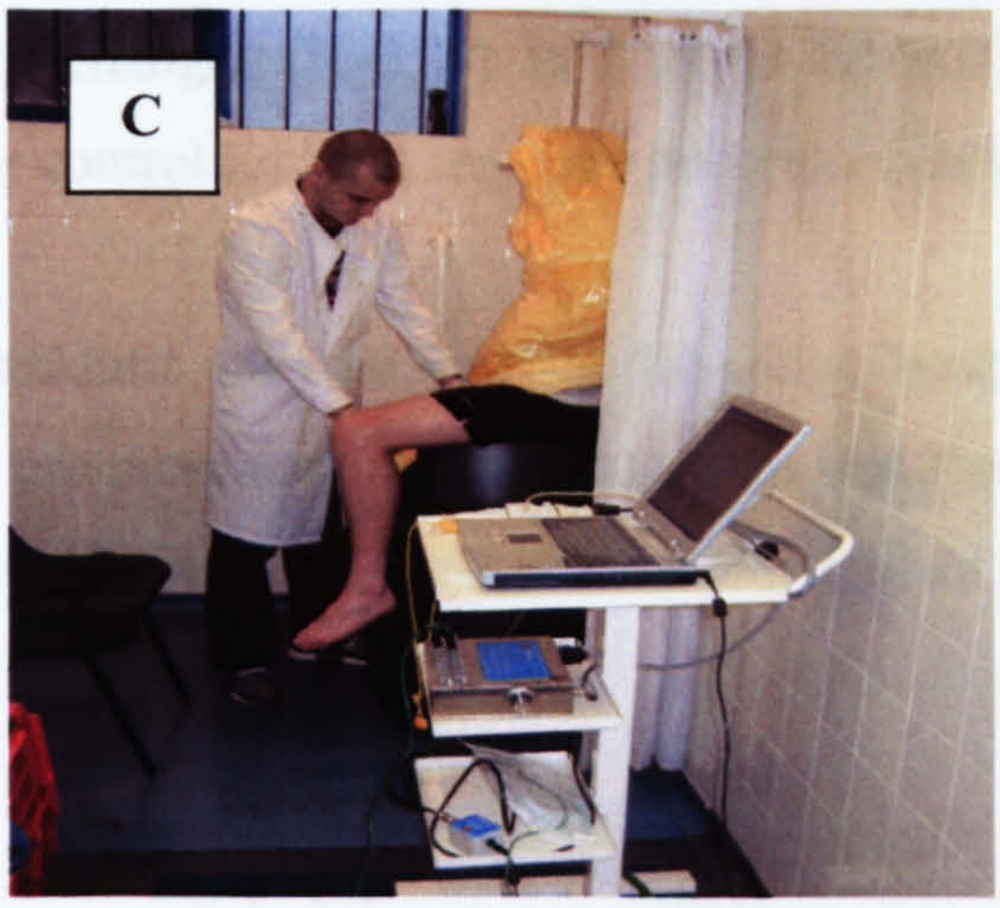


Figure 6.1 – Illustration of the experimental set-up. (A) Subjects immersed one limb in the heated water tank and rested their contra-lateral limb over the side of the tank. (B) Subjects wore a T-shirt throughout the heating protocol and also wrapped their head and torso in plastic lining so as to prevent heat loss from the core and head. (C) Core temperature was monitored continuously during the protocol via an on line analysis system.

6.3 RESULTS

6.3.1 Physiological and thermoregulatory responses to the heating protocol. Subjects' heart rate and ratings of thermal comfort during the heating protocol are shown in Figure 6.2. Both heart rate and ratings of thermal comfort showed a significant increase ($P<0.05$) during heating which became significantly different from baseline at 10 mins following the start of the protocol. Muscle temperature of the heated and control limbs during the experimental protocol are shown in Figure 6.3A. Muscle temperature of the heated limb showed a significant increase ($P<0.05$) during heating, increasing from 35.9 ± 0.2 °C at baseline to 39.5 ± 0.2 °C upon completion of the 60 min heating protocol. In contrast, muscle temperature of the control limb showed no significant change ($P>0.05$) from baseline at any time-point during the experimental protocol (pre: 36.1 ± 0.2 °C; post: 35.7 ± 0.2 °C). The heating protocol also induced a significant increase ($P<0.05$) in core temperature, increasing from 37.4 ± 0.2 °C at baseline to 38.9 ± 0.2 °C post-heating (Figure 6.3B). This increase in core temperature was significantly different ($P<0.05$) from baseline after 10 mins of heat exposure.

6.3.2 Muscle HSPs and MnSOD content following heating. The muscle HSP and MnSOD protein content of the heated and control limbs are presented in Figure 6.4 A-F. No significant changes ($P>0.05$) in HSP or MnSOD protein content were observed in heated or control limbs at any time point examined.

6.3.3 Muscle antioxidant enzyme activity following heating. Muscle total SOD and catalase activity are presented in Figure 6.5. No significant changes ($P>0.05$) in activity of these enzymes were observed in heated or control limbs at any time point examined.

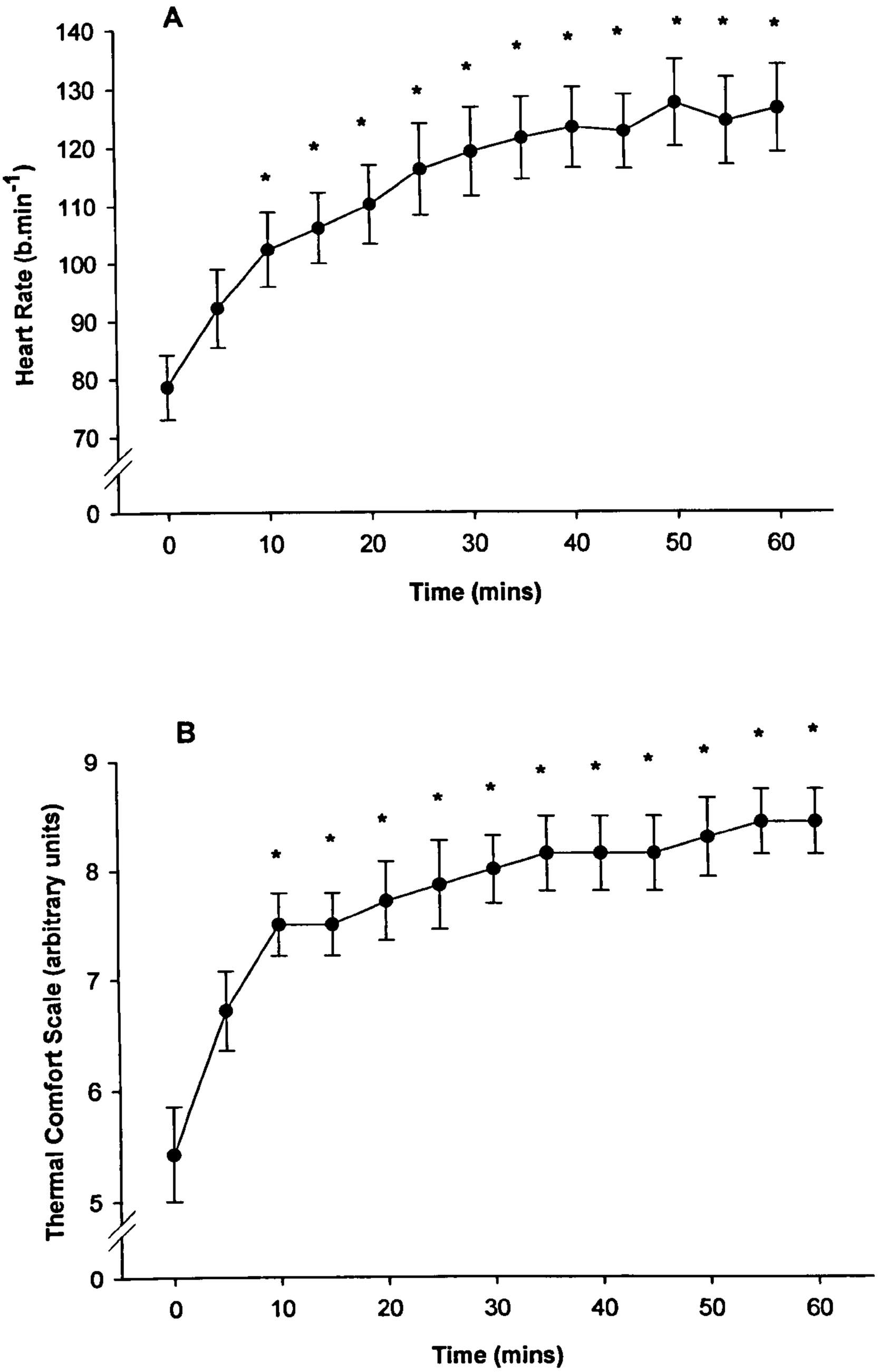


Figure 6.2 – (A) Heart rate and (B) ratings of thermal comfort during the experimental protocol. * denotes significant difference from pre-heating values, $P<0.05$.

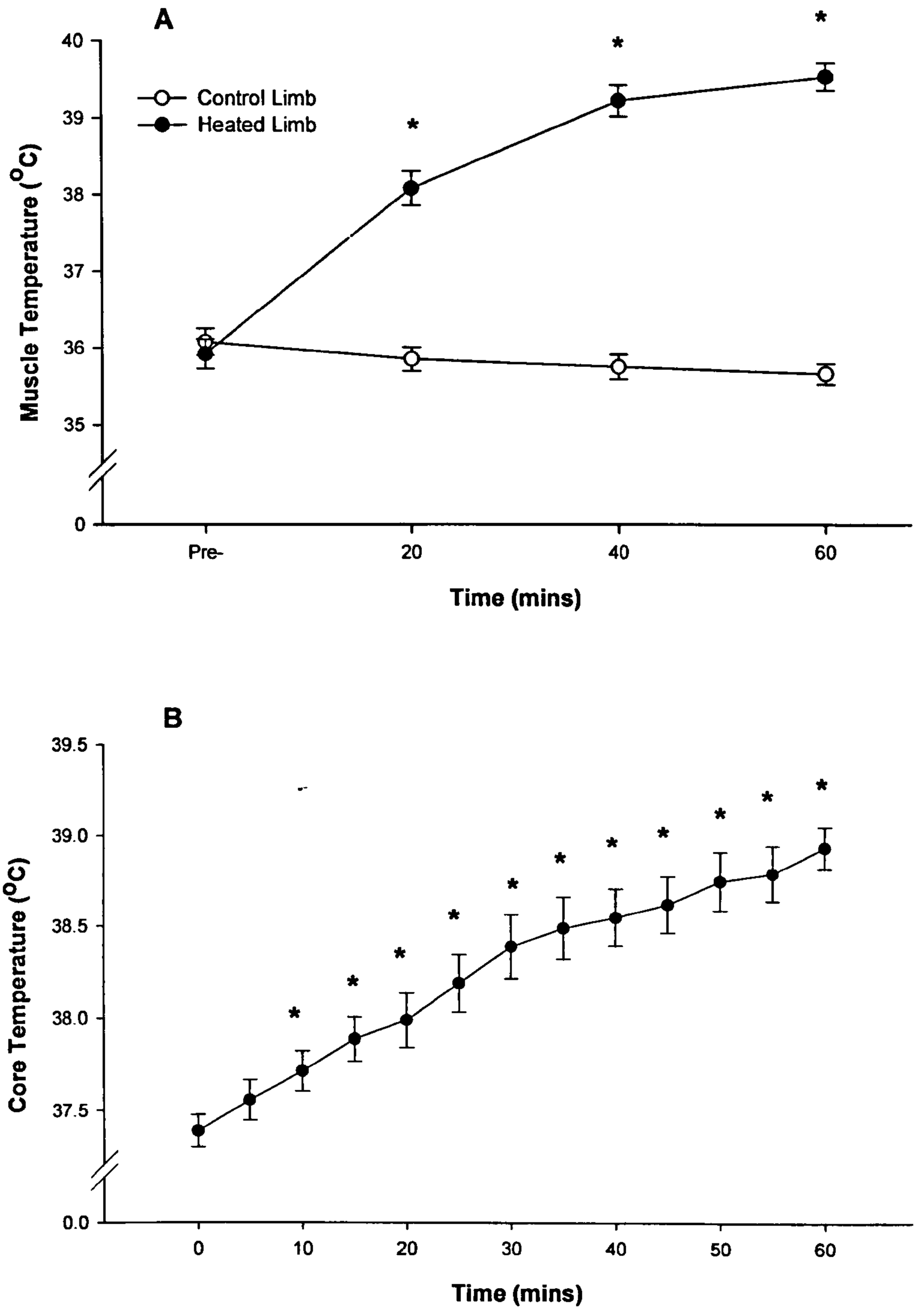


Figure 6.3 – (A) Muscle temperature of the vastus lateralis of control and heated limbs during the experimental protocol. * denotes significant difference from pre-experimental values and from control limb values at the corresponding time-point, $P<0.05$. (B) Core temperature during the heating protocol. * denotes significant difference from pre-heating values, $P<0.05$.

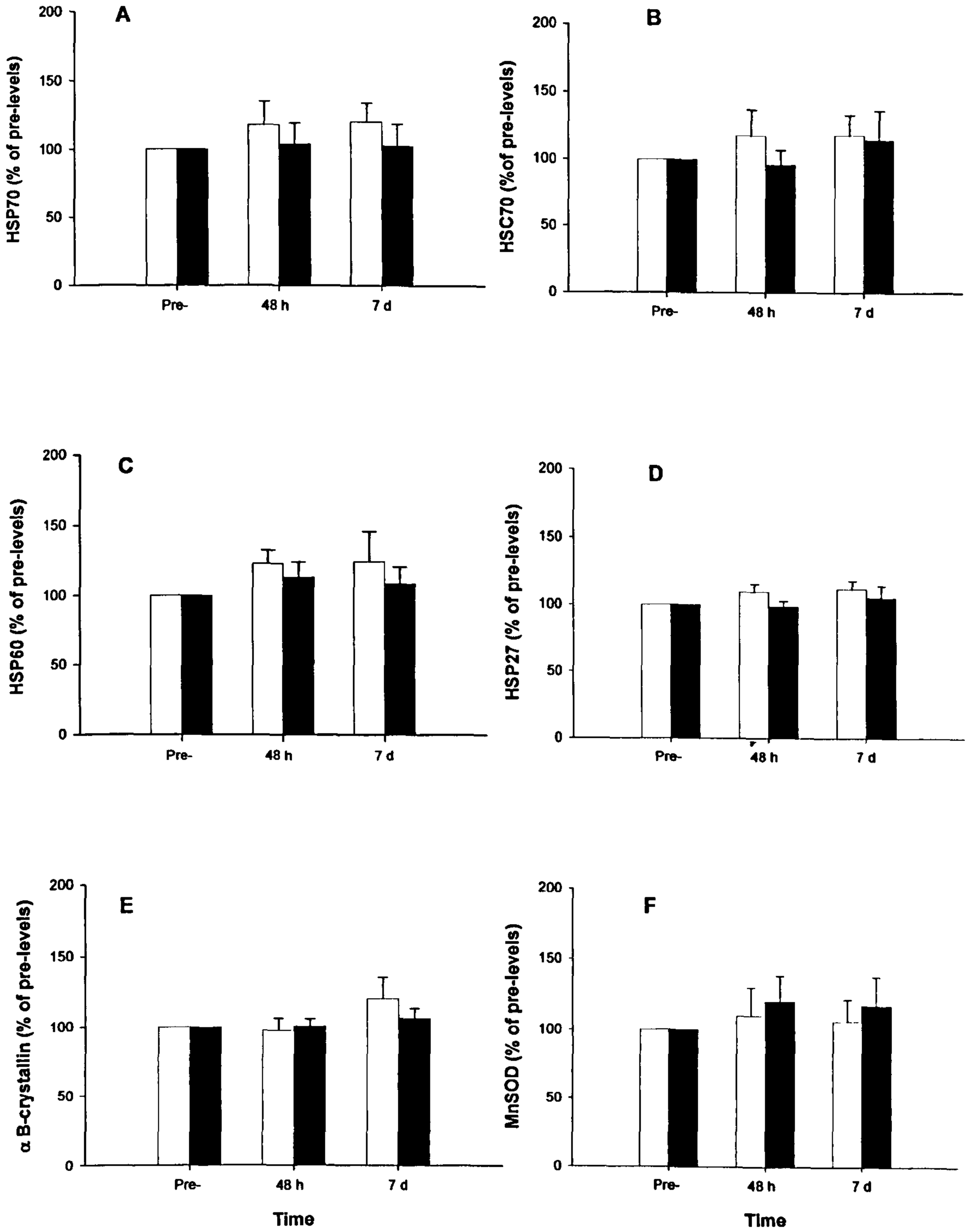
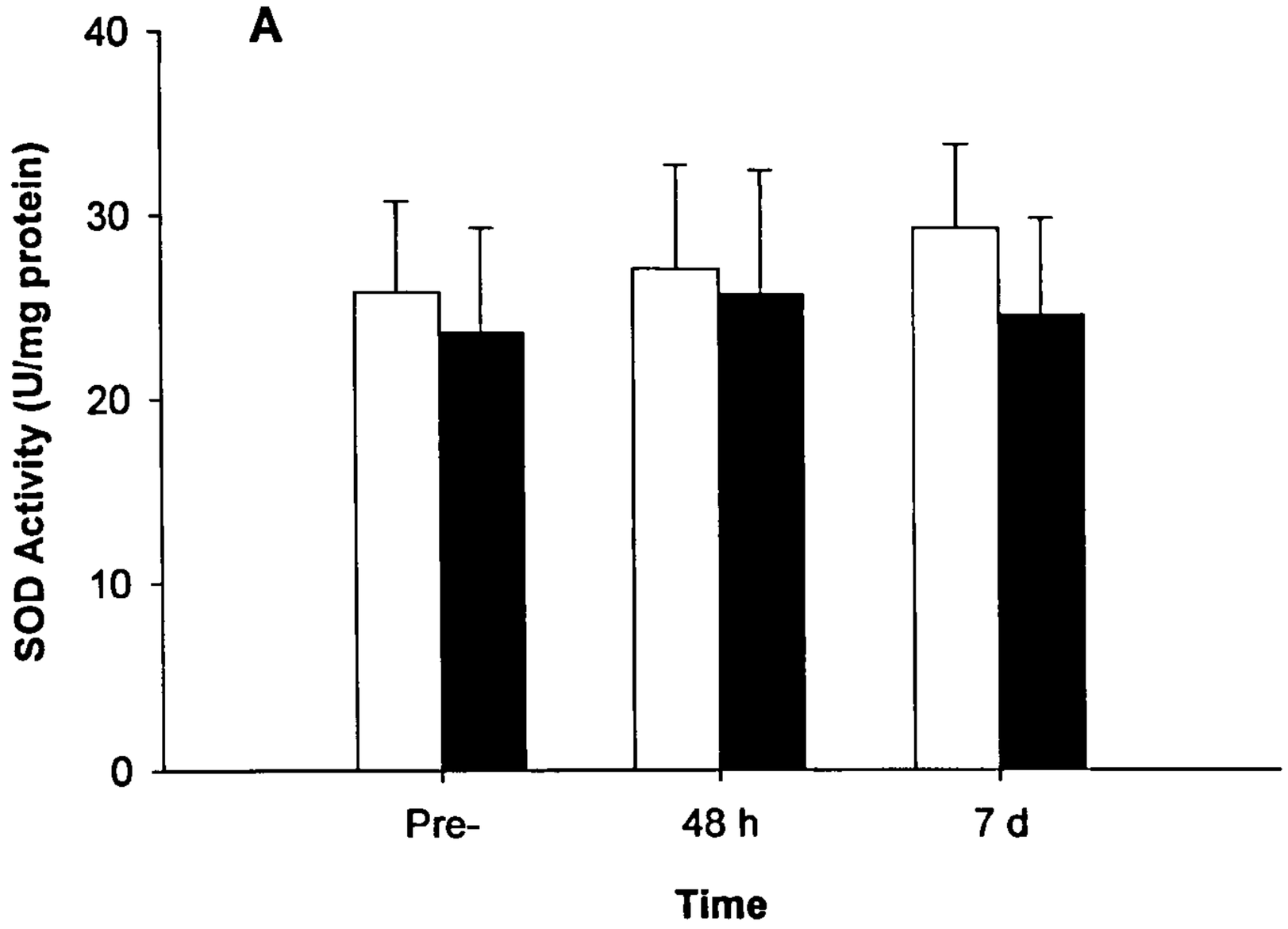


Figure 6.4 – (A) HSP70, (B) HSC70, (C) HSP60, (D) HSP27, (E) αB-crystallin and (F) MnSOD content of the vastus lateralis of the control (white bars) and heated limbs (black bars) following the experimental protocol.



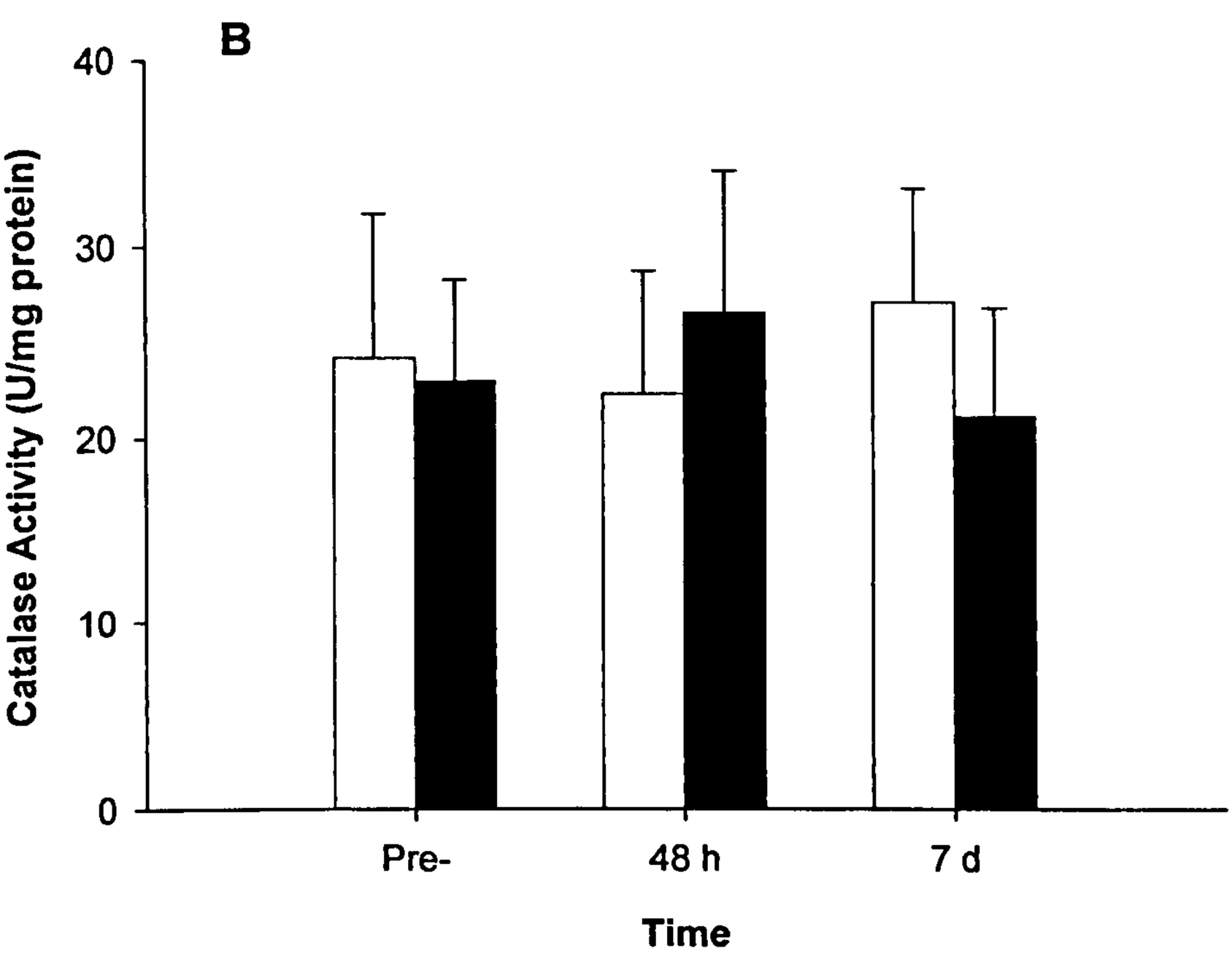


Figure 6.5 – (A) Total SOD and (B) catalase activity of the vastus lateralis of control (white bars) and heated (black bars) limbs following the experimental protocol.

6.4 DISCUSSION

The aim of the present study was to examine the effect of a passive heating protocol on HSP content in human skeletal muscle. By elevating both muscle and core temperature to a similar extent as that typically observed during moderately demanding aerobic exercise (Study 3), the role of hyperthermia in contributing to the exercise-induced production of HSPs was evaluated. This study provides novel data for the literature and is the first to investigate the heat shock response of human skeletal muscle *in vivo* following a thermal stress of physiological relevance. Contrary to the author's hypothesis, no significant increase was observed in any of the HSPs examined following heating, suggesting that exercise-induced increases in core and muscle temperature *per se* do not appear to be the major cellular stressor that results in the exercise-induced production of HSPs in human skeletal muscle.

The novel heating protocol employed here is advantageous as it replicates the systemic and local thermal challenge of exercise whilst also allowing for comparison of heated and control muscle from the same individual. Similar models have been previously employed in rodent studies where a rise in muscle temperature to 42°C induced a significant 1.5 - 2.2 fold increase in HSP60, HSP70 and HSC70 in soleus and gastronemius muscles (Oishi *et al.*, 2002, 2003). It is acknowledged that similar elevations in HSP content may be possible in human skeletal muscle if muscle temperature is increased to similar levels. However, whilst this temperature is physiologically relevant to the exercising rodent (Brooks *et al.*, 1971) and similar to those temperatures used to induce HSP expression *in vitro* (Mizzen and Welch, 1998, Maglara *et al.*, 2003; Fehrenbach *et al.*, 2000), it is both ethically challenging and physiologically irrelevant for the exercising human.

Several other studies are in agreement with the present data in that elevations in muscle and core temperature may not be the sole factor responsible for inducing increased production of muscle HSPs following exercise. Using a rodent model, Skidmore *et al.* (1995) observed increases in HSP70 content in both skeletal and cardiac muscle after 1 h of treadmill running which were independent of an increase in core temperature. Electrical stimulation protocols have also demonstrated increases in muscle HSP content in the absence of increased muscle temperatures

(Neufer *et al.*, 1996; McArdle *et al.*, 2001). The magnitude of HSP increases observed in rodent studies following treadmill running (Locke *et al.*, 1990; Salo *et al.*, 1991; Skidmore *et al.*, 1995; Hernando and Manso, 1997; Milne and Noble, 2002) or electrical stimulation protocols (Neufer *et al.*, 1996; McArdle *et al.*, 2001) are also somewhat larger than that observed following *in vivo* heating protocols (Oishi *et al.*, 2002, 2003) suggesting that additional factors may be contributing to the exercise-induced production of HSPs. These data, in combination with the present findings, strongly suggest that non-heat stress factors associated with muscle contractile activity are therefore of more importance than elevated temperature *per se* in contributing to the exercise-induced production of HSPs in both animal and human skeletal muscle.

Exercise is associated with an array of other disruptions to cellular homeostasis, many of which have been shown to induce HSP expression *in vitro*. Experimental evidence for a role of substrate availability in influencing the HSP72 response to acute exercise has been demonstrated (Febbraio *et al.*, 2002b) and several studies now suggest that redox disturbances in muscle homeostasis may be the dominant signalling mechanism in activating the exercise-induced stress response (McArdle *et al.*, 2001; Khassaf *et al.*, 2003; Jackson *et al.*, 2004; Fischer *et al.*, 2006). In relation to this hypothesis, it is noteworthy to consider that hyperthermia in itself causes increased generation of ROS (Salo *et al.*, 1991; Zuo *et al.*, 2000) which may thereby lead to increased HSP production. However, whilst a hyperthermia mediated increase in ROS production may contribute to increased HSP expression in rodent models employing whole body exercise (e.g. treadmill running), the present data demonstrate that this pathway appears not to be responsible for the exercise-induced production of HSPs in human skeletal muscle as muscle temperature is rarely elevated above 40°C. In such cases, the stress response is likely mediated by those ROS produced from muscle contraction, the precise source and species of which remain to be elucidated.

Indeed, McArdle *et al.* (2001) observed increases in muscle HSP content (in the absence of elevated muscle temperature) following non-damaging contractions in hindlimb muscles of mice which was preceded by an increased superoxide production during contractions and a transient and reversible oxidation of protein thiols at 15 min

post-stimulation. In agreement with data from other cell types (Freeman *et al.*, 1995), it was postulated that exercise resulted in increased production of ROS and an accompanying reversible oxidation of muscle proteins which was part of a key signalling mechanism inducing the adaptive up-regulation of HSPs. Further evidence for a role of exercise generated ROS in initiating the stress response is provided by a series of antioxidant supplementation studies where the increased production of HSP70 following 1-legged cycle ergometry in humans was abolished following vitamin C (Khassaf *et al.*, 2003), vitamin E (Jackson *et al.*, 2004) or β-carotene supplementation (Jackson *et al.*, 2004). Recent data suggests that this elevation of tissue antioxidant capacity directly scavenges exercise generated ROS thereby abolishing transcriptional activation of the HSP genes (Fischer *et al.*, 2006). These authors observed that the combination of vitamin C and E supplementation inhibited increases in muscle HSP72 mRNA expression immediately following 3 h of knee extensor exercise. Trained rodents also exhibited a diminished HSP70 response to customary exercise than untrained rodents, which was suggested to be due to an increased baseline antioxidant defence system following training (Smolka *et al.*, 2000). Together, these data demonstrate, unequivocally, a role of ROS in activation of the exercise-induced stress response of human skeletal muscle. Further research is required, however, to elucidate the precise mechanisms underlying this response.

In summary, the present data demonstrate that elevations of core and muscle temperature to similar levels as that typically occurring during aerobic exercise fails to induce a significant increase in protein content of the major HSP families in human skeletal muscle. It is concluded that the exercise-induced elevations in muscle HSP content previously observed by us and others are not due to exercise-associated increases in systemic or local muscle temperature *per se*. In light of accumulating data, it is therefore suggested that such a response may be mediated by exercise-induced redox signalling in order to allow the tissue to adapt to the oxidative stress generated by exercise and to confer increased protection against subsequent stresses.

Chapter 7

The influence of training status on basal HSP levels and the magnitude of the exercise-induced stress response of human skeletal muscle

The work presented in this Chapter aimed to examine the influence of training status on the muscle content of those major HSP families examined in previous Chapters. This work also examined the influence of training status on the magnitude of the exercise-induced stress response following acute non-damaging running exercise.

7.1 INTRODUCTION

The exercise-induced stress response of human skeletal muscle displays remarkably high individual variation in terms of both magnitude and time-course of the response. This appears to be due, in part, to individual differences in baseline HSP levels (Study 3; Walsh *et al.*, 2001; Khassaf *et al.*, 2001). Typically, those individuals with relatively low levels of HSP70 respond to exercise with a faster (i.e. 24 – 48 h exercise) and larger increase in HSP70 expression whereas subjects with higher baseline levels tend to exhibit a slower (i.e. 72 h – 7 days post-exercise) and much smaller response. In considering factors that determine basal HSP levels and / or the extent of the heat shock response to various stresses, training status (Gonzalez *et al.*, 2000), recent activity levels (Campisi *et al.*, 2003), thermal history (Kregel, 2002), predominant fibre type (O'Neill *et al.*, 2006) and energy availability (Febbraio *et al.*, 2002b) have all been considered as possible determinants. Given that all of the above are partly dependent upon training status *per se*, it is possible that much of the variability reported previously, both within and between studies, may therefore be related to subtle differences in training status between subjects. Indeed, whilst the subjects employed in Study 3 were predominantly 'active' in nature, individual differences in physical fitness criteria were also evident.

An increase in baseline muscle HSP content with exercise training has consistently been observed in rodent muscle (Gonzalez *et al.*, 2000; Mattson *et al.*, 2000; Naito *et al.*, 2001; Samelan, 2000). In addition to training-induced adaptations of basal HSP status, it has also been suggested that exercise training might allow an individual to mount a greater and/or faster stress response following an acute exercise stress (Campisi *et al.*, 2003; Gonzalez *et al.*, 2000). Gonzalez *et al.* (2000) observed that when trained rodents performed an acute bout of treadmill running (of lower intensity than the customary training sessions) 3 days after their last training session, the ratio of their post-exercise vs resting synthetic rates of HSP72 was significantly elevated compared to sedentary controls. Campisi *et al.* (2003) also demonstrated that habitual physical activity facilitates a larger stress-induced production of HSP72 in brain, peripheral and immune tissues following a novel tail shock stress or exhaustive treadmill running exercise. These data therefore suggest that trained individuals may

exhibit a larger production of HSPs to an acute exercise stress, which may subsequently facilitate a more rapid and successful recovery. Alternatively, it is possible that the HSP response to acute exercise in the trained state is associated with a reduced stress response as the muscle is already pre-conditioned with appropriate endogenous defence systems (Smolka *et al.*, 2000). These authors observed that trained rodents exhibit a diminished HSP70 response to customary exercise compared with untrained rodents, a finding attributed to a training-induced increase in baseline antioxidant defences (Smolka *et al.*, 2000).

Studies employing short-term training interventions in humans have typically demonstrated that increases in HSP70 content observed in the initial stages of training are only maintained if the training stimulus is held constant or increased (Liu *et al.*, 1999, 2000). When the training stimulus is reduced, it appears that HSP levels may return to baseline values. These data therefore suggest that many of the reported training-induced increases in basal HSP levels may simply reflect the change in exercise intensity/duration from the previous acute exercise session. Furthermore, the subjects used in these investigations were already of elite nature and hence it is possible that the short-term training interventions were insufficient to induce further gross changes in muscle HSP levels. As such, the pre-training intervention levels of HSPs in these subjects may have already been appropriate to counteract the stress of customary training routines and indeed may already be considerably higher than those of less conditioned or sedentary individuals. It would therefore seem appropriate to also employ cross-sectional designs and examine the basal level of HSPs in skeletal muscle of both untrained and trained individuals who are in a rested state. Such designs allow comparison of muscle HSP levels from subjects with distinct physiological differences and may therefore help explain some of the individual variability that appears inherent of the exercise-induced stress response.

The effects of exercise training on muscle HSP status also warrants investigation because of the enhanced protection conferred to skeletal muscle against stress following an increased content of HSPs (Welch, 1992; Garramone *et al.*, 1994; Lepore *et al.*, 2000; Maglara *et al.*, 2003; A. McArdle *et al.*, 2004; F.McArdle *et al.*, 2004). For example, A.McArdle *et al.* (2004) demonstrated the EDL muscle of transgenic adult and aged mice overexpressing HSP70 is significantly protected

against damage induced by lengthening contractions. A consistent elevation of basal content of HSPs during repeated exercise may therefore be a crucial component of the cellular and molecular mechanisms underpinning the increased protection to contraction-induced damage associated with exercise training (Locke and Noble, 2002).

The aim of the present study was to therefore examine the baseline levels of HSPs and aspects of antioxidant defences in the skeletal muscle of trained and untrained humans. In an attempt to determine how training status influences the magnitude of the stress response following exercise, both subject populations also performed a non-damaging bout of running exercise as described previously. It was hypothesised that trained subjects would exhibit an increase in baseline HSP and antioxidant protein levels / enzyme activity and would therefore display a diminished stress response to acute exercise compared with the untrained subjects studied here and the active population examined previously in Study 3.

7.2 METHODS

7.2.1 Subjects. Twelve healthy males volunteered to participate in the study (mean ± SD: age, 29 ± 6 years; weight, 77.1 ± 11.3 kg; height, 1.78 ± 0.04 m). The study was approved by the Ethics Committee of Liverpool John Moores University and all subjects conformed to the criteria outlined in section 3.1.2.

7.2.2 Design. Subjects were initially assessed for $\dot{V}O_{2max}$ (see section 3.3) and lactate threshold (see section 3.4). These tests were separated by at least 48 h. Subjects were subsequently divided into a 'trained' (n=6) and 'untrained' (n=6) group according to the criteria outlined in section 3.1.2. Having then refrained from exercise and prolonged thermal exposure (i.e. baths, saunas, steam rooms, tanning devices) for at least 4-5 days following the lactate threshold test, subjects completed a 45 min treadmill running protocol at a speed corresponding to their lactate threshold (see section 3.8). Muscle biopsies were obtained from the vastus lateralis (see section

3.10) immediately prior to the exercise protocol and at 48 h and 7 days post-exercise (these time-points were selected on the basis of our time-course approach in Study 3 examining the stress response following acute non-damaging running exercise). Muscle samples were analysed for HSP70, HSC70, HSP60, HSP27, αB-crystallin and MnSOD protein content as well as activity of total SOD (see section 3.11-3.12).

In order to ascertain that the exercise protocol was also non-damaging for untrained subjects, two additional untrained subjects performed the exercise protocol where measurements of maximal quadriceps isometric force and venous blood samples were obtained prior to and at 2 h, 6 h, 24 h, 48 h and 72 h post-exercise. Similar to the active and trained males in Study 2, the exercise protocol resulted in no significant decrements in maximal quadriceps isometric muscle force and in clinically insignificant increases in circulating creatine kinase levels and subjective ratings of perceived muscle soreness (data not shown).

7.2.3 Statistical analyses. Differences in baseline physical and physiological characteristics and in resting muscle content of HSPs and antioxidant protein / enzyme activity were assessed using a students *t*-test for independent samples. Changes in exercise related variables during the exercise protocol and changes in muscle HSP content and antioxidant protein content/enzyme activity following exercise were analysed using a two-way mixed design repeated measurements General Linear Models where the 'within subject' factor was time and 'between subject' factor was training status. Where there was a significant main effect for time, paired *t*- tests with Bonferroni corrections were used for post-hoc analysis. All data are presented as means ± SD with P values of <0.05 indicating statistical significance.

7.3 RESULTS

7.3.1 Baseline physical and physiological characteristics. Baseline physical and physiological baseline characteristics of the untrained and trained groups are displayed in Table 7.1. There was a significant difference ($P<0.05$) between groups in $\dot{V}O_{2max}$, lactate threshold (both % of $\dot{V}O_{2max}$ and running speed at LT) and number of hours involved in physical activity per week.

Table 7.1 – Physical and physiological characteristics of the untrained and trained subject groups.

	UNTRAINED	TRAINED
Age (years)	29 ± 6	28 ± 6
Height (m)	1.76 ± 0.05	1.79 ± 0.03
Weight (kg)	78.1 ± 15.5	76.1 ± 6
$\dot{V}O_{2max}$ ($ml.kg^{-1}.min^{-1}$)	48.9 ± 2.9	64.2 ± 4.6 *
LT (% of $\dot{V}O_{2max}$)	64.5 ± 1.9	76.1 ± 2.9 *
Running speed @ LT ($km.h^{-1}$)	9.4 ± 1	13.8 ± 0.3 *
Hours of activity per week	0.7 ± 0.8	7.7 ± 1.5 *

*denotes significant difference between groups, $P<0.05$.

7.3.2 Baseline muscle HSPs and MnSOD levels. Baseline muscle HSP and MnSOD levels of untrained and trained subjects are displayed in Figure 7.1. HSP70 and HSC70 content showed a tendency to increase in trained subjects with non-significant increases of 13 and 16% over untrained muscle, respectively ($P = 0.08$ and 0.09, respectively). Muscle content of αB-crystallin in trained subjects showed a significant increase ($P<0.05$) of 43 % over untrained subjects. In contrast, there was no significant difference in muscle HSP27 content between subject groups. HSP60 and MnSOD content in trained subjects also displayed a significant increase ($P<0.05$) of 25 and 45% over untrained muscle, respectively.

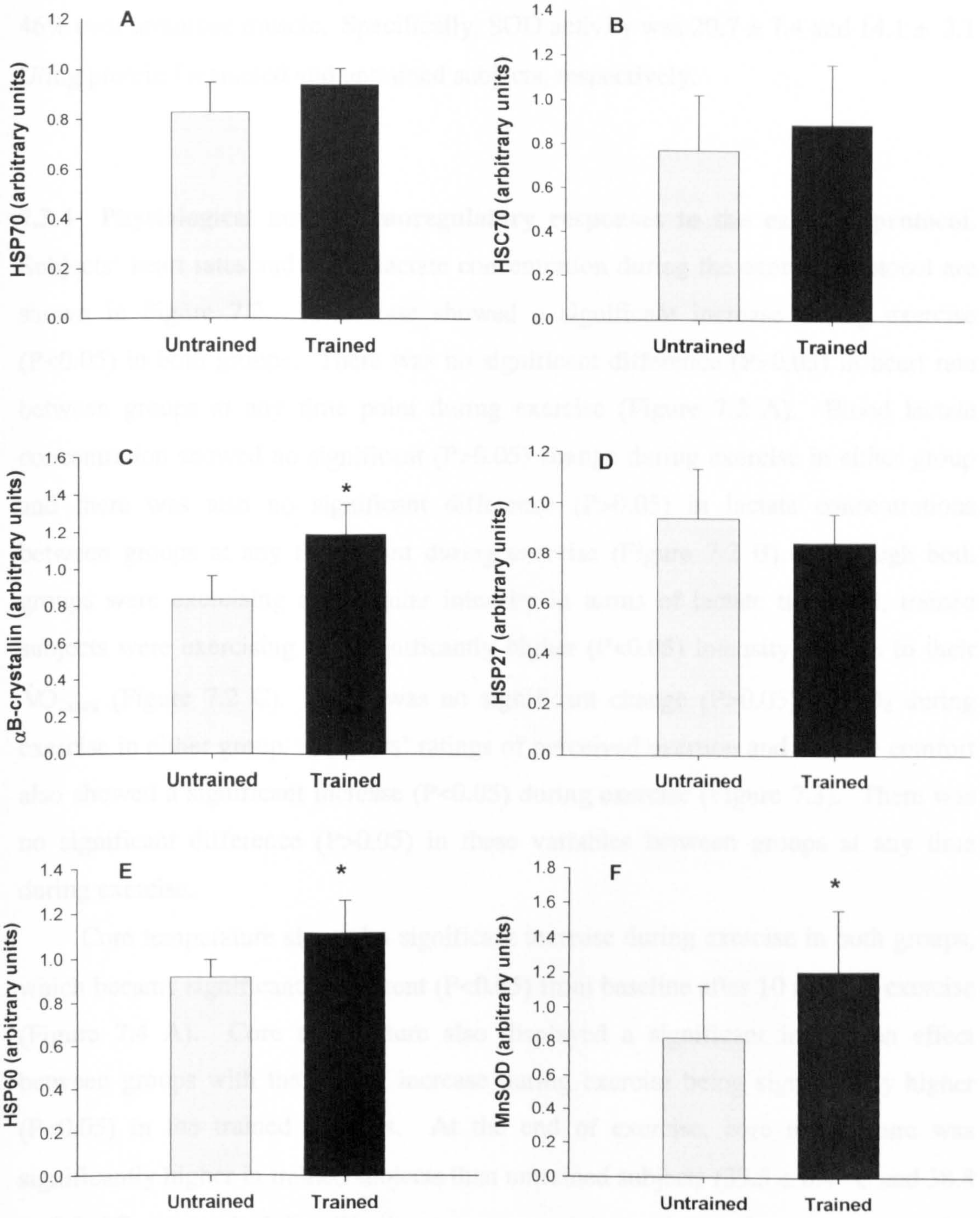


Figure 7.1 – Baseline protein content of (A) HSP70, (B) HSC70, (C) αB-crystallin, (D) HSP27, (E) HSP60 and (F) MnSOD of the vastus lateralis of untrained and trained subjects. * denotes significant difference between subject groups, $P<0.05$.

7.3.3 Baseline muscle SOD activity. Baseline total SOD activity showed a tendency (P = 0.08) to increase in trained muscle, with a non-significant increase of 46% over untrained muscle. Specifically, SOD activity was 20.7 ± 7.4 and 14.1 ± 3.1 U/mg protein for trained and untrained subjects, respectively.

7.3.4 Physiological and thermoregulatory responses to the exercise protocol. Subjects' heart rates and blood lactate concentration during the exercise protocol are shown in Figure 7.2. Heart rate showed a significant increase during exercise (P<0.05) in both groups. There was no significant difference (P>0.05) in heart rate between groups at any time point during exercise (Figure 7.2 A). Blood lactate concentration showed no significant (P>0.05) change during exercise in either group and there was also no significant difference (P>0.05) in lactate concentrations between groups at any time point during exercise (Figure 7.2 B). Although both groups were exercising at a similar intensity in terms of lactate threshold, trained subjects were exercising at a significantly higher (P<0.05) intensity relative to their $\dot{V}O_{2max}$ (Figure 7.2 C). There was no significant change (P>0.05) in $\dot{V}O_2$ during exercise in either group. Subjects' ratings of perceived exertion and thermal comfort also showed a significant increase (P<0.05) during exercise (Figure 7.3). There was no significant difference (P>0.05) in these variables between groups at any time during exercise.

Core temperature showed a significant increase during exercise in both groups, which became significantly different (P<0.05) from baseline after 10 mins of exercise (Figure 7.4 A). Core temperature also displayed a significant interaction effect between groups with the rate of increase during exercise being significantly higher (P<0.05) in the trained subjects. At the end of exercise, core temperature was significantly higher in trained subjects than untrained subjects (39.5 ± 0.4 °C and 38.8 ± 0.5 °C, respectively). Similar to core temperature, muscle temperature also significantly increased following exercise (P<0.05) and displayed a significant interaction effect (P<0.05) between groups (Figure 7.4 B). Muscle temperature was significantly higher in trained subjects than untrained subjects immediately post-exercise (40.5 ± 0.4 °C and 39.8 ± 0.4 °C, respectively).

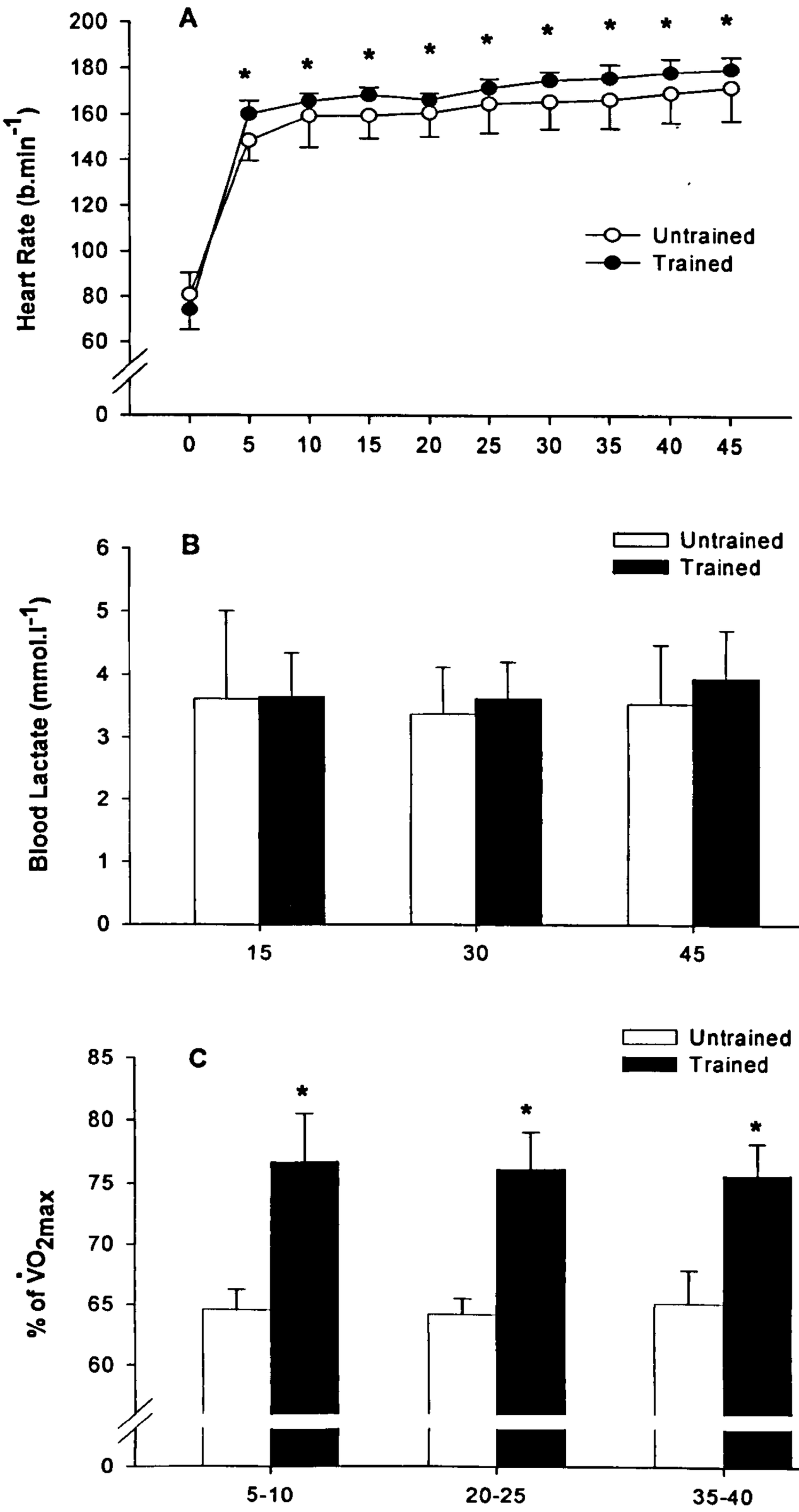


Figure 7.2 – (A) Heart rates of untrained and trained subjects during the exercise protocol, * denotes significant difference from pre-exercise values, $P<0.05$. (B) Blood lactate concentrations of untrained and trained subjects during the exercise protocol. (C) Exercise intensity, in terms of percentage of $\dot{V}O_{2max}$, of the untrained and trained subjects during the exercise protocol, * denotes significant difference between groups, $P<0.05$.

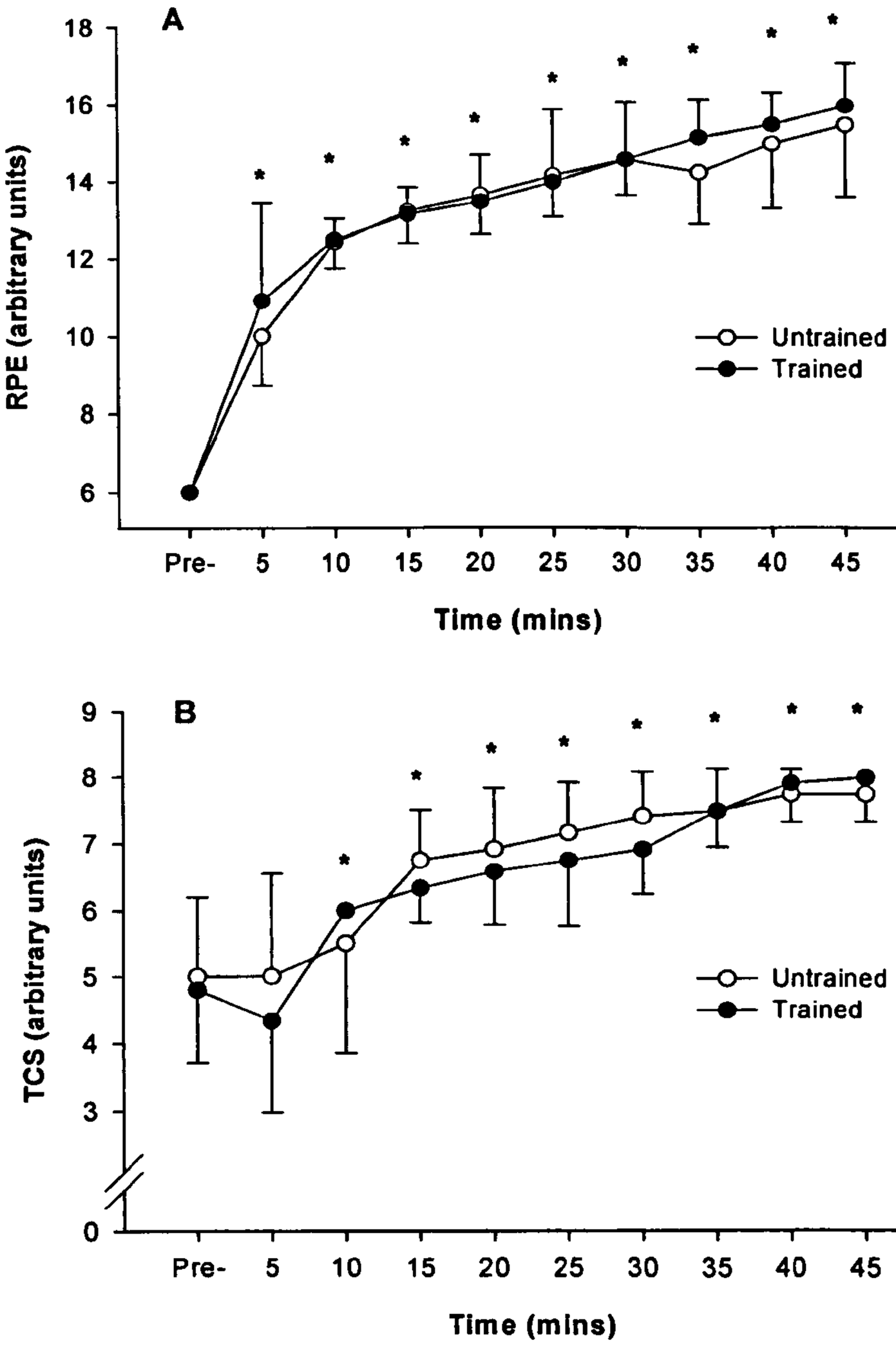


Figure 7.3 – Untrained and trained subjects' ratings of (A) perceived exertion and (B) thermal comfort during the exercise protocol. * denotes significant difference from pre-exercise values.

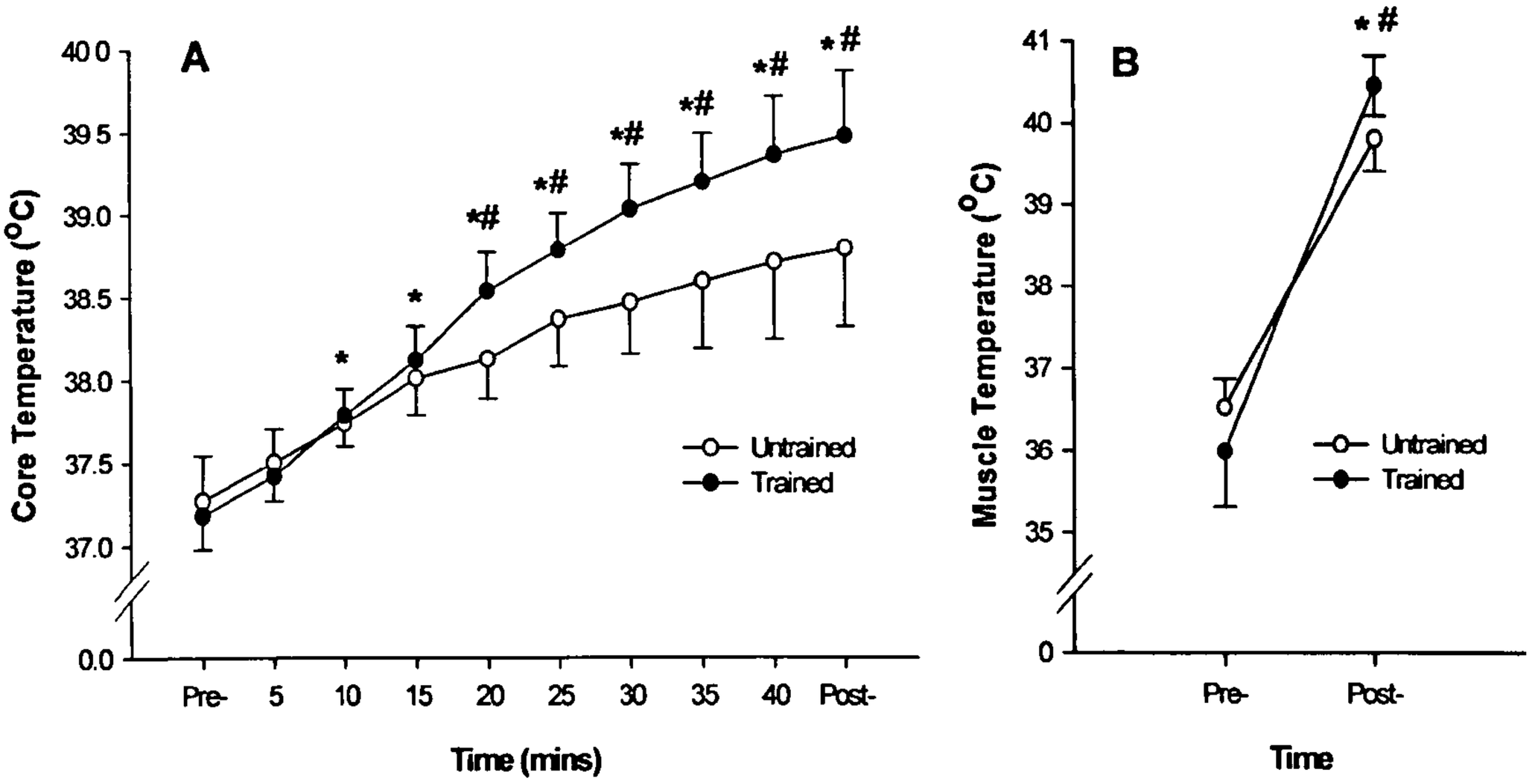


Figure 7.4 – (A) Core temperature of the untrained and trained subjects during the exercise protocol and (B) muscle temperature of the vastus lateralis of the untrained and trained subjects pre- and post-exercise. * denotes significant difference from pre-exercise values, $P<0.05$. # denotes significant interaction effect, $P<0.05$.

7.3.5 Muscle HSPs and MnSOD content following exercise. Muscle HSP and MnSOD content of the untrained and trained subjects following exercise are presented in Figure 7.5. No significant changes ($P>0.05$) in HSP or MnSOD protein content were observed in either group at any time point following the exercise protocol.

7.3.6 Muscle SOD activity following exercise. Total SOD activity following the exercise protocol is shown in Figure 7.6. Total SOD activity displayed no significant ($P>0.05$) change in either group following exercise.

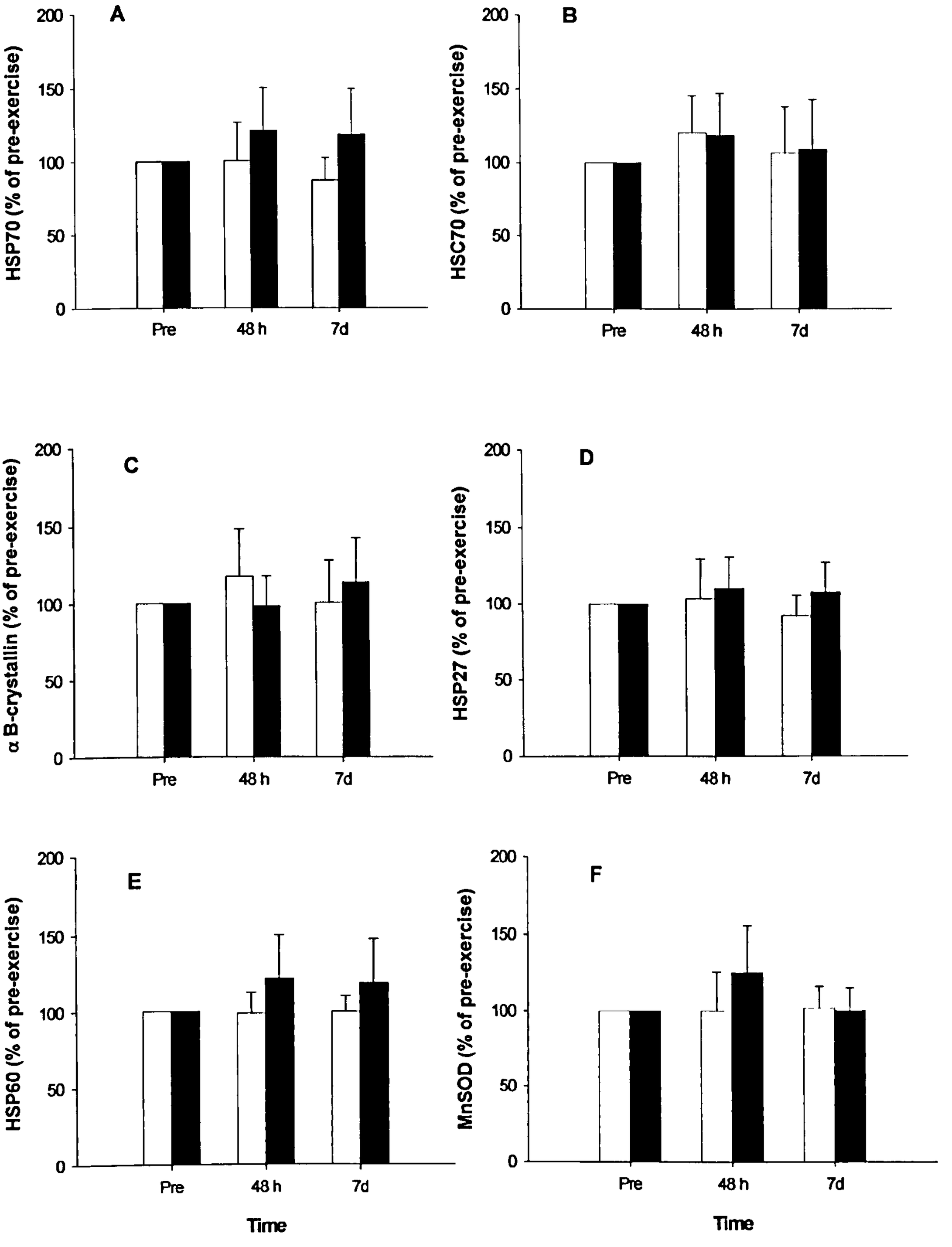


Figure 7.5 – (A) HSP70, (B) HSC70, (C) αB-crystallin, (D) HSP27, (E) HSP60 and (F) MnSOD content of the vastus lateralis of untrained (white bars) and trained (black bars) subjects following exercise.

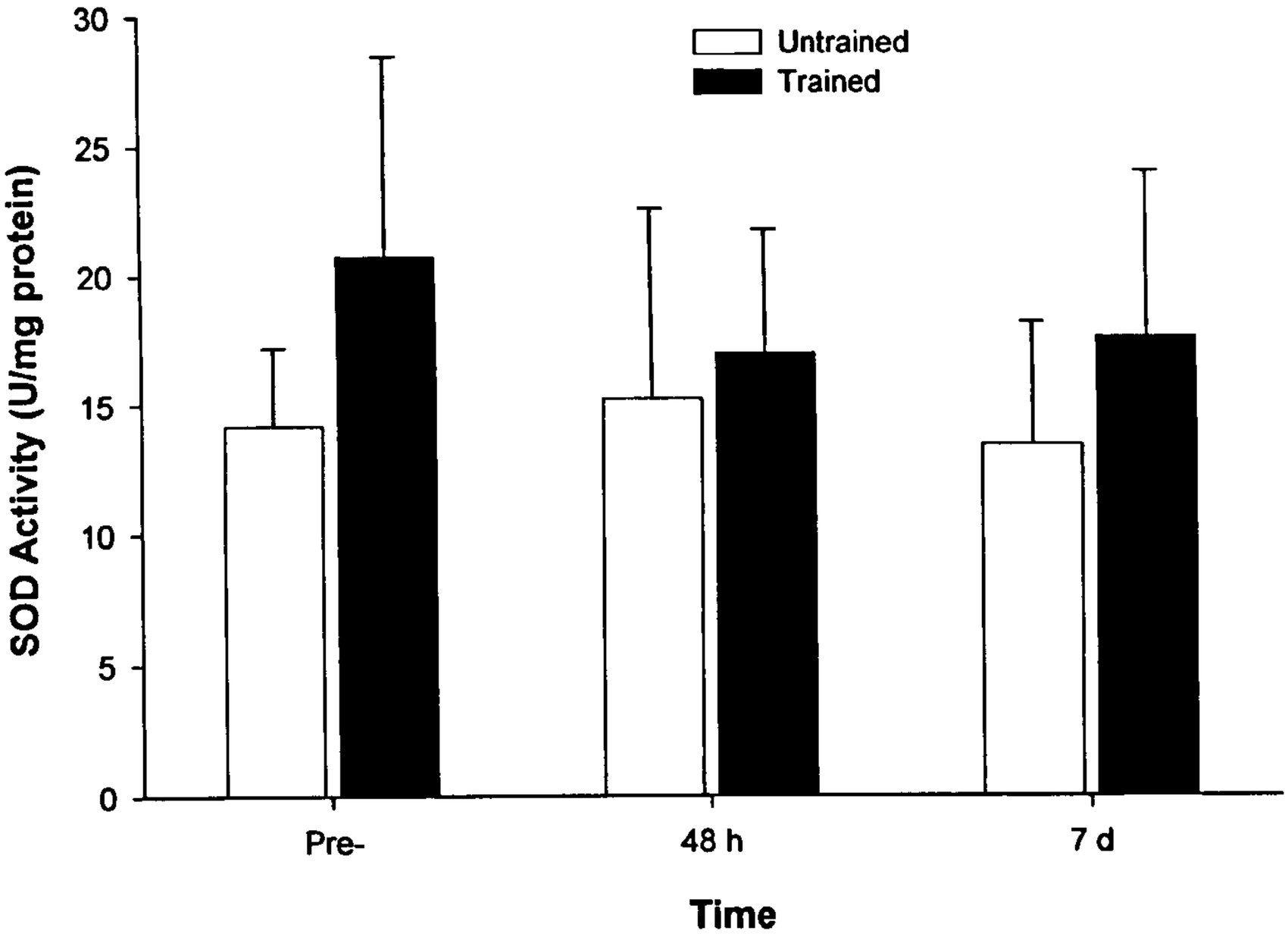


Figure 7.6 – Total SOD activity of the vastus lateralis of untrained and trained subjects following exercise.

7.4 DISCUSSION

The aim of the present study was to investigate the influence of training status on basal HSP levels and also on the magnitude of the stress response to an acute exercise stress. Using a cross-sectional design, data demonstrate for the first time that the vastus lateralis muscle of trained subjects displays an enhanced baseline protective system with up-regulation of several HSPs and antioxidant defence proteins than compared with untrained subjects. When compared with the active subject population studied previously (Study 3), neither the trained nor untrained subjects examined here exhibited a stress response to acute exercise. These data suggest that some of the individual variability in basal HSP levels and the exercise-induced production of HSPs observed previously (Study 3; Khassaf *et al.*, 2001; Walsh *et al.*, 2001) may therefore be related to individual differences in training status and its related components.

The response of basal muscle HSP levels to exercise training is particularly important given their potential cytoprotective properties. Whilst data from rodent studies has consistently shown an increase in HSP content following periods of endurance training (Gonzalez *et al.*, 2000; Mattson *et al.*, 2000; Naito *et al.*, 2001; Samelan, 2000), the limited data available from human studies are less convincing and have been restricted to HSP70. Liu *et al.* (2000) demonstrated that the increases in HSP70 observed during the initial stages of a short-term rowing training program are only maintained if the training stimulus is either held constant or increased. When the training intensity was reduced, muscle HSP70 levels returned to pre-training values. In the present study, there was a tendency for basal HSP70 ($P = 0.08$) and HSC70 ($P = 0.09$) to increase in trained muscle with increases of 13 and 16%, respectively. These data may therefore be in agreement with Liu *et al.* (2000) in that significant increases in muscle HSP70 content may only represent an acute response in order to facilitate adaptation to a stress of a novel homeostatic disruption. When the muscle recovers from the acute stress, HSP70 may return to baseline levels. Indeed, the trained subjects in the present study had not performed their habitual training routines for at least 7 days prior to their resting muscle biopsy. Alternatively, it is possible that training status does significantly affect resting HSP70 levels but that the present

sample size (n = 6) was underpowered to achieve significance. It was difficult to perform detailed *a-priori* power calculations, however, because of uncertainties of the variance of HSP content/antioxidant enzyme activity in these populations under resting conditions or of meaningful effect sizes. Nevertheless, the present data provide a sound framework for further research in that detailed statistical power calculations can now be performed.

In relation to the small HSPs, αB-crystallin content was significantly higher in trained muscle compared with untrained muscle. These findings confirm and extend those of Yoshioka *et al.* (2003) who observed an increased muscle αB-crystallin gene expression in endurance trained subjects when compared with sedentary individuals. In contrast, HSP27 showed no significant difference between trained and untrained muscle. The apparent selective up-regulation of αB-crystallin with no accompanying increase in HSP27 is intriguing and warrants further investigation. The small HSPs are thought to interact with cytoskeleton relating proteins following exposure to stress (Mounier and Arrigo, 2002). It has therefore been suggested that small HSPs may be particularly important following damaging forms of exercise where they function to assist in the maintenance and remodelling of myofibrillar structures (Koh, 2002; Feasson *et al.*, 2002). Using a transgenic model, McArdle *et al.* (personal communication) demonstrated that the skeletal muscle of mice overexpressing αB-crystallin displays an increased protection to the contraction-induced damage that is evident at 3 h following lengthening contraction. Furthermore, Melkani *et al.* (2006) recently demonstrated that αB-crystallin maintains skeletal muscle myosin ATPase activity and prevents its aggregation under heat shock stress. Based on the above observations, it is therefore suggested that the observed increase of αB-crystallin in trained muscle has an important physiological role which functions to protect and repair the cytoskeleton and contractile machinery during the stress of daily training and ultimately maintain muscle performance.

The present data also demonstrate an increased baseline content of HSP60 in trained vs untrained muscle. These data agree with findings from animal studies in that chronic contractile activity (Ornatsky *et al.*, 1995; Neufer *et al.*, 1996) and periods of treadmill training (Samelman, 2000; Mattson *et al.*, 2000) also up-regulate muscle

HSP60 content. The up-regulation of HSP60 in trained muscle reported here is likely of physiological relevance in facilitating protein import and protein folding during exercise-induced mitochondrial biogenesis and also in protecting mitochondrial proteins against damage during exercise (Hood *et al.*, 2000).

The significant increase in MnSOD protein content in trained muscle was accompanied by a tendency ($P = 0.08$) for increased total SOD activity. This apparent adaptation of antioxidant defences likely functions to maintain redox balance and reduce the potentially damaging effects of ROS during everyday exercise. These data provide novel data for the literature and appear consistent with findings from rodent muscle where a training-induced increase in both enzyme protein and activity is well documented (Higuchi *et al.*, 1985; Ji *et al.*, 1988; Oh-ishi *et al.*, 1997Ji, 1999; Powers and Lennon, 1999). Data concerning the response of antioxidant defences of human skeletal muscle to exercise training, however, are conflicting and extremely limited. Whereas short-term training protocols have failed to induce an increase in SOD activity (Hellsten *et al.*, 1996; Tiidus *et al.*, 1996), Jenkins *et al.* (1984) observed an increased enzyme activity in 'high' aerobic capacity males ($\dot{V}O_{2max}$ 65 ± 2 $ml^{-1}.kg^{-1}.min^{-1}$) compared with 'low' aerobic capacity males ($\dot{V}O_{2max}$ 51± 2 $ml^{-1}.kg^{-1}.min^{-1}$). As such, the impact of acute and chronic exercise on ROS production and antioxidant defences of human skeletal remains poorly defined and therefore warrants further investigation.

In comparison to the active subject population studied previously (Study 3), neither subject population studied here exhibited a stress response to the exercise protocol. The absence of a stress response in trained muscle confirms the author's hypothesis and is likely due to the increase in baseline protective systems (discussed above) and the customary nature of the exercise protocol, which was representative of daily training routines. Indeed, all of the trained subjects were accustomed to such laboratory exercise protocols and had recently trained for and competed in prolonged endurance events (e.g. marathon running). The observation of a blunted stress response to customary exercise in trained muscle has previously been demonstrated in rodent muscle in which the authors also attributed to an increase in baseline protective systems such as an increased antioxidant enzyme activity (Smolka *et al.*, 2000).

Similar data are also available from humans where a group of well trained rowers did not show any increase in muscle HSP70 gene expression within 6 hours following a high intensity strength training session or low intensity endurance training session (Nething *et al.*, 2004). It is possible, therefore, that whereas the stress response following an acute exercise stress functions to restore homeostasis, the stress of repeated exercise results in adaptations that serve to maintain homeostatic balance during everyday training activity.

Contrary to the author's hypothesis, the untrained subjects also did not display a stress response to acute exercise. This finding is attributed to the low intensity nature of the exercise protocol for this population. Despite being matched for lactate threshold, the protocol equated to the lower absolute exercise intensity 60-65% of $\dot{V}O_{2max}$ and treadmill speeds of somewhere between brisk walking and gentle jogging pace. These data therefore suggest that there are important characteristics regarding the relative or absolute exercise intensity which are extremely important in influencing the stress response to exercise. It was initially chosen to match exercise intensity between groups for lactate threshold based on observations that exercise at this intensity elicits similar relative circulatory and metabolic strain irrespective of training status (Baldwin *et al.*, 2000). However, considering that the absolute treadmill speeds were significantly lower for untrained subjects (i.e. gentle jog above walking pace vs moderate running pace), it is likely that fibre recruitment patterns and the total fibre recruitment during exercise would be considerably different between groups. This may be of particular importance for activation of the exercise-induced stress response (Milne and Noble, 2002).

Data from rodent muscle have demonstrated a critical threshold exercise intensity that is required to elicit increases in muscle HSP70 content following acute treadmill running (Milne and Noble, 2002). These authors further highlighted that this critical intensity is dependent on known muscle recruitment patterns. For example, HSP70 displayed significant increases in the soleus muscle following running at low to moderate speeds only (15 to 27 m/min) whereas it exhibited a significant increase in the red and white portions of the vastus muscle only when exercise was performed at the highest running speeds (27 to 33 m/min). These data suggest that the exercise-

induced production of HSPs is therefore heavily dependent on an absolute workload which should be sufficient to recruit a significant proportion of the muscle fibres. It is difficult to extrapolate these findings to a human model because the vastus lateralis is a predominantly mixed fibre type. It is therefore impossible to isolate the fibre source of increased HSP content in whole muscle homogenate using western blotting procedures. Nevertheless, it is suggested that the absence of a stress response in the untrained subjects may simply be due to an inadequate absolute workload that is required to exceed the critical threshold of necessary fibre recruitment.

Had the exercise intensity of both groups been matched relative to $\dot{V}O_{2max}$, it is likely that these subjects would have displayed a clear stress response given that the absolute work load would be much more similar between groups. In this situation, the less conditioned muscle of untrained subjects would then therefore experience an increase in total fibre recruitment (particularly that of type 2) and greater metabolic stress (e.g. increased ROS production, carbohydrate utilisation etc) so as to exercise at the higher workload. These subjects may thereby need to exhibit a larger production of HSPs in order combat the increased homeostatic disruption to protein structure and function. Indeed, less conditioned muscle of sedentary subjects exhibits a clear HSP70 response to acute 1-legged cycling when exercising at the higher absolute workload of 70% $\dot{V}O_{2max}$ (Khassaf *et al.*, 2001, 2003; Jackson *et al.*, 2004).

Taken together, these data highlight a possible exercise intensity dependent relationship on muscle HSP production whereby a critical threshold intensity exists beyond which exercise induces an increase in HSP levels. It is further proposed that this critical threshold exercise intensity is dependent on individual training status (and related characteristics such as baseline defence systems) and the novelty of the exercise task. These ideas are discussed further in Chapter 8. Although an exercise-intensity dependent relationship on HSP production has previously been demonstrated in rodent muscle (Milne and Noble, 2002) and in the circulation of human subjects (Fehrenbach *et al.*, 2005) following acute exercise, no such relationship has been substantiated in human muscle following an acute exercise stress. Further work is needed to therefore clarify these issues.

In summary, the present data demonstrate that exercise training is associated with a selective up-regulation of several HSPs and antioxidant defence proteins in human skeletal muscle. The up-regulation of such defence systems likely functions to maintain homeostasis during the stress of daily training by protecting against disruptions to the cytoskeleton and contractile machinery, maintaining redox balance, facilitating mitochondrial biogenesis and contributing to the overall remodelling of the cell. Neither the trained nor untrained groups studied here exhibited an increased HSP production following the exercise protocol. The absence of a stress response in both populations suggests that HSPs are up-regulated in an exercise intensity dependent fashion that is dependent upon attainment of a critical exercise intensity threshold and is further complicated by individual training status and overall muscle fibre recruitment. Further studies are required to examine this possible exercise intensity dependent relationship on muscle HSP production.

Chapter 8

Synthesis of Findings

8. SYNTHESIS OF FINDINGS

The aim of this Chapter is to review and integrate the experimental findings from Studies 1 – 5. The Chapter begins by briefly revisiting the aims and objectives of the thesis and reviewing if each, in turn, have been achieved. A general discussion of findings is then presented and important methodological limitations are also discussed. Following an overall conclusion, the thesis closes by outlining recommendations for future research.

8.1 ACHIEVEMENT OF AIMS AND OBJECTIVES

Aim 1: *To develop a non-damaging running exercise protocol to utilise as an exercise stimulus to initiate the expression of HSPs in human skeletal muscle, where non-damaging is defined as exercise that results in no overt structural or functional damage to the muscle.*

This aim was achieved via completion of Studies 1, 2, and 5. In Study 1, the reliability of maximal quadriceps isometric force and voluntary activation were established across a time-scale for how these variables were to be assessed in Study 2. Using both active and trained subjects, Study 2 demonstrated that 45 min of running exercise on a motorised treadmill at an intensity corresponding to the lactate threshold results in no significant decrement in maximal quadriceps isometric force and voluntary activation and in clinically insignificant increases in circulating creatine kinase levels and ratings of perceived muscle soreness. Similar data were also obtained for untrained male subjects in Study 5. This protocol was therefore deemed non-damaging in nature and was considered suitable to use as an exercise stimulus to investigate the exercise-induced stress response of skeletal muscle in future studies.

Aim 2: *To characterise the time-course and magnitude of response of the major HSP families in human skeletal muscle following an acute bout of non-damaging running exercise.*

This aim was achieved via completion of Study 3. Using an active male population, the data presented in this study demonstrated that non-damaging running exercise results in a differential up-regulation of specific HSPs where peak responses typically occur at 48 h post-exercise. These data also highlighted an individual variation of baseline muscle content of HSPs and in the magnitude of the exercise-induced production of HSPs.

Aim 3: *To evaluate the role of increases in muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle.*

This aim was achieved via completion of Study 4. This study demonstrated that elevations of muscle and core temperature to similar levels as those observed during the exercise protocol in Study 3, resulted in no significant increases in HSP content in the skeletal muscle of active male subjects. It was therefore suggested that exercise-induced hyperthermia does not play a major role in contributing to the exercise-induced production of HSPs in human skeletal muscle.

Aim 4: *To determine the influence of training status on the baseline content of the major families of HSPs in human skeletal muscle.*

This aim was achieved via the completion of Study 5. Using a cross sectional design, this study demonstrated that trained muscle displays a selective up-regulation of several HSPs compared with untrained muscle.

Aim 5: *To determine the influence of training status on the magnitude of the exercise-induced stress response of human skeletal muscle following an acute bout of non-damaging running exercise.*

This aim was achieved via the completion of Study 5. This study demonstrated that trained and untrained muscle does not exhibit a stress response to acute exercise.

8.2 General Discussion of Findings

The studies undertaken in this thesis were designed to investigate the exercise-induced production of HSPs in human skeletal muscle. Specifically, they were intended to evaluate the role of elevated muscle and core temperature in contributing to the exercise-induced production of HSPs and to also examine the influence of training status on the basal levels of HSPs and on the magnitude of the exercise-induced stress response. These studies have provided novel data for the literature and have significantly advanced our understanding of the exercise-induced stress response of human skeletal muscle.

Prior to beginning experimental work, data concerning the exercise-induced stress response of human skeletal muscle were extremely limited and confusing. It was difficult to draw definitive conclusions from these data because of important methodological differences between studies e.g. timing of biopsy sampling, specific HSP measured, disparate exercise protocols, muscle group examined and differing inter-subject characteristics. An increase in several HSPs had been observed in the vastus lateralis following 1-legged cycle ergometry (Khassaf *et al.*, 2001, 2003; Jackson *et al.*, 2004), knee extensor exercise (Febbraio *et al.*, 2002b) and downhill running (Feasson *et al.*, 2002) and in the biceps following lengthening contractions (Thompson *et al.*, 2001, 2002, 2003).

It has been suggested that interpretation of data is particularly complicated in those instances where there is a damaging component to the exercise protocol because of the inflammatory response that accompanies such exercise (Vasilaki *et al.*, 2006). In order to avoid the complications of changes in cell type (e.g. phagocytic cell content) on interpretation of data, it has therefore been recommended that non-damaging exercise protocols provide a more controlled methodological approach for which to study the exercise-induced regulation of HSP expression *in vivo* in both rodents (McArdle *et al.*, 2001Vasilaki *et al.*, 2006) and humans (Khassaf *et al.*, 2001, 2003; Jackson *et al.*, 2004).

The present thesis has extended these findings by developing and employing a non-damaging running exercise protocol to induce increases in muscle HSP content of the vastus lateralis (Study 1 and 2). Running was chosen as the exercise protocol because of the relevance to the general population and in an array of sporting activities. Furthermore, prior to beginning experimental work, data concerning the stress response of skeletal muscle following running exercise was extremely limited (Punstchart *et al.*, 1996; Walsh *et al.*, 2001). The characterisation of 'non-damaging' was based on the definition that the exercise protocol induces no overt structural or functional damage to the muscle of untrained, active or trained subjects, as evidenced by indirect indicators of muscle damage such as circulating CK levels and maximal isometric muscle force. It is acknowledged, however, that in order to conclusively ascertain an exercise protocol as non-damaging it is necessary to perform histological analysis using light or electron microscopy on muscle biopsy samples obtained immediately pre- and in the days following exercise. Such procedures were not undertaken here owing to cost, technical and time constraints. However, based on the CK and force data, it is unlikely that histological analysis would have revealed any evidence of gross necrosis or gross infiltration of phagocytic cells (Vasilaki *et al.*, 2006).

Previous studies that employed running exercise to investigate the stress response failed to detect any increase in HSP content of the vastus lateralis of active male subjects within several hours following 30 min of running exercise at the anaerobic threshold (Punstchart *et al.*, 1996) or 1 h of running at 70% $\dot{V}O_{2max}$ (Walsh *et al.*, 2001). Both studies observed an increased HSP70 gene expression, however, suggesting that the exercise intensity had been sufficient to activate the appropriate signalling pathway. It was therefore speculated that exercise-induced translation of HSPs in human skeletal muscle may require greater than 24 h, somewhat longer than that typically needed in rodent muscle (Locke *et al.*, 1990; Salo *et al.*, 1991; Hernando and Manso, 1997; McArdle *et al.*, 2001). The above data therefore suggest that multiple biopsy samples obtained beyond 24 h post-exercise are needed in order to accurately determine the time-course of the response.

Surprisingly, only one study thus far has attempted to characterise the time-course of the response using a serial biopsy approach where biopsy samples were obtained beyond 24 h post-exercise (Khassaf *et al.*, 2001). This is likely due, in part, to the difficulties facing investigators in relation to the acquisition of sufficient tissue and also the significant discomfort experienced by subjects. In exercise physiology research, muscle biopsies have been traditionally obtained using the Bergstrom or Conchotone needle biopsy techniques. In the present thesis, the Pro-Mag biopsy gun technique was used for obtaining samples. The rationale for this technique was based on subjects' previous experiences that this technique was somewhat less traumatic and is significantly more 'subject friendly'. This feedback was crucial in the choice of biopsy procedure and was particularly important for study design in the present thesis given the serial muscle biopsies that are needed to investigate the stress response of skeletal muscle. Whilst this technique is limited in that it yields significantly less tissue than traditional approaches, it is also highly advantageous for those researchers interested in obtaining serial biopsies where limited or specific analyses are required. It should also be noted, however, that the process of muscle biopsies (in general) is limited in that the small amount of tissue sampled may not be representative of the whole muscle. This is especially the case with the vastus lateralis muscle where data are often difficult to interpret due to the mixed fibre composition of the muscle (Johnson *et al.*, 1973).

The present thesis has adopted the above technique to characterise the time-course and magnitude of responses of the major HSP families in the vastus lateralis muscle of an active male subject population following an acute bout of non-damaging running exercise (Study 3). Importantly, this study examined the response of multiple HSPs in contrast to much of the previous research which focused solely on the response of HSP70. These data demonstrate, for the first time, that the stress of an acute bout of moderately demanding running exercise is sufficient to induce up-regulation of HSP70, HSC70 and HSP60. Whilst this response appeared variable in both time-course and magnitude of the response, examination of individual data revealed that peak increases typically occur at 48 h post-exercise. In contrast, exercise failed to induce an up-regulation of the small HSPs of HSP27 and αB-crystallin. It was therefore concluded that acute exercise induces a differential

expression of specific HSPs according to certain characteristics of the particular exercise protocol. Based on these data and related research (Feasson *et al.*, 2002), it was suggested that the HSP70 and HSP60 proteins may be up-regulated via oxidative, thermal, metabolic or cytokine signals whereas the small HSPs are more responsive to contractile-induced mechanical disruptions to protein structure.

Although Study 3 characterised the time-course of the exercise-induced stress response of human skeletal muscle, it did not provide any comprehensive analysis as to the possible signals initiating the up-regulation of HSPs. The exercise protocol did induce significant increases in both rectal and muscle temperature suggesting that exercise associated hyperthermia may at least, in part, contribute to the exercise-induced production of HSPs. In order to address this hypothesis, Study 4 subsequently evaluated the role of elevated muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle. In this study, a similar cohort of subjects to that employed in Study 3 were subjected to a 1 h passive heating stress during which muscle and core temperature were increased to similar levels as those observed during exercise in Study 3. It was demonstrated that elevations of muscle and core temperature did not result in any significant increase in muscle content of any of the HSPs examined. These data suggest that exercise associated hyperthermia *per se* does not appear to be the major stressor that results in the exercise-induced production of HSPs. Further evidence against a role of elevated temperature was provided in Study 5 in which trained subjects failed to exhibit a stress response, despite significantly higher post-exercise core and muscle temperatures than those observed in active subjects following exercise or heating.

There are now several lines of evidence suggesting that exercise-induced ROS formation and an accompanying oxidation of protein sulphydryl groups is the dominant signalling mechanism that induces an up-regulation of HSP content following exercise (McArdle *et al.*, 2001; Khassaf *et al.*, 2001, 2003; Jackson *et al.*, 2004; Fischer *et al.*, 2006). Unfortunately, none of the classic biochemical hallmarks of oxidative stress / signalling were measured in the present thesis owing to the lack of available tissue and inappropriate timing of biopsy sampling. In relation to this hypothesis, it would have therefore been beneficial to perform additional biopsies immediately post- and within several hours post-exercise so as to provide an insight to

the degree of oxidative stress / signalling induced by exercise. Such samples could have been analysed for increased ROS production using both direct (e.g. electron spin resonance) and indirect methods such as muscle total glutathione levels, oxidised glutathione levels, protein thiols and protein carbonyls etc. These samples could have also been analysed for HSF1 activation and HSP mRNA so as to provide an insight as to the temporal relationship between redox signalling, transcriptional activation of the HSP genes and increased muscle HSP content. These ideas are discussed further in section 8.4 as a recommendation for future research.

The exercise-induced production of HSPs in human skeletal muscle displays individual variation in both the time-course and magnitude of the stress response. This was demonstrated in Study 3 and also in previously published data (Khassaf *et al.*, 2001; Walsh *et al.*, 2001). This variation appears to be due, in part, to individual differences in resting HSP levels prior to exercise. For example, subjects with lower baseline HSP levels displayed an earlier and more pronounced stress response whereas those subjects with higher resting levels of HSPs tended to exhibit a delayed and much smaller stress response. This individual variation in basal HSP levels may be related to differences in training status which may subsequently influence the magnitude of the exercise-induced stress response. Indeed, factors such as recent activity levels (Campisi *et al.*, 2002), thermal history (Kregel, 2002), resting glycogen content (Febbraio *et al.*, 2002a), predominant fibre type (O'Neill *et al.*, 2006) which are inherent characteristics of training status can all influence constitutive HSP expression and/or the magnitude of the heat shock response to various stresses.

The present thesis has presented novel data demonstrating that individual differences in training status appear to influence both basal HSP levels and the magnitude of the exercise-induced stress response (Study 5). Using a cross-sectional design employing six trained and six untrained subjects, Study 5 demonstrated an increased protein content of αB-crystallin and HSP60 and a tendency ($P<0.1$) for increased HSP70 and HSC70 levels in skeletal muscle of trained subjects compared with untrained individuals. In relation to antioxidant defences, trained subjects also showed a higher protein content of MnSOD and a tendency for increased total SOD activity ($P<0.1$). This up-regulation of baseline defence systems likely functions to maintain

homeostasis during the stress of daily training by protecting against disruptions to the cytoskeleton and contractile machinery, maintaining redox balance, facilitating mitochondrial biogenesis and contributing to the overall remodelling of the muscle cell. For those variables that only showed a tendency to be increased in trained muscle, it is possible that these data may have been underpowered to detect statistical significance and that larger samples would have been needed. It was difficult to perform detailed *a-priori* power calculations, however, because of uncertainties of the variance of HSP content/antioxidant enzyme activity in these populations under resting conditions or of meaningful effect sizes. Nevertheless, the present data provide a sound framework for further research in that detailed statistical power calculations can now be performed.

In addition to subject sample sizes, it is also of particular importance to ensure appropriate and distinct subject classification criteria when comparing HSP status between populations with differing training backgrounds. All of the subjects employed in the present thesis were described as untrained, active or trained young healthy males according to their $\dot{V}O_{2max}$, lactate threshold and number of hours involved in physical activity per week. Whilst the author is confident that appropriate subject classification was achieved (group data demonstrated distinct significant differences between all pair-wise comparisons for each descriptive variable; see Table 3.1), there were also individual cases in which several subjects overlapped according to certain criteria. This was particularly the case for $\dot{V}O_{2max}$. For example, some untrained subjects displayed $\dot{V}O_{2max}$ values that may be considered representative of an active population although their lactate threshold or time involved in physical activity per week did not conform to those values typically observed in active subjects. This was also the case when comparing certain active subjects with trained subjects. Given the well documented variability in $\dot{V}O_{2max}$ values and the array of data demonstrating velocity at lactate threshold as the best indicator of endurance performance (for review see Basset and Howley, 2000), it was therefore considered appropriate to place more importance on lactate threshold and physical activity data when classifying these particular subjects.

Although the groups used in Study 5 were of distinct physiological difference, the untrained population were by no means representative of a sedentary population, as evidenced by $\dot{V}O_{2max}$ and physical activity data. When comparing basal levels of HSPs, it may have therefore been appropriate to also include sedentary subjects in the population sample. It is, however, extremely difficult to recruit subjects from the sedentary population who are willing to participate in the muscle biopsy procedure. Furthermore, it is highly unlikely that these subjects would have been able to perform the exercise part of the study. Nevertheless, it is recommended that subjects representative of a sedentary population be included in future cross-sectional designs if possible.

The observation of enhanced baseline protective systems in trained muscle leads to the obvious conclusion that consistent elevations of HSPs during repeated exercise may be one of multiple mechanisms by which exercise training can provide protection to cells and tissues against related and non-related stressors. Transgenic approaches in rodents have demonstrated HSP mediated protection against ischemia-reperfusion injury in the heart (Marber *et al.*, 1995) or against contraction induced injury to skeletal muscle (A.McArdle *et al.*, 2004). Pre-conditioning exercise stresses have also demonstrated similar effects in rodent tissue (Locke *et al.*, 1995b; F.McArdle *et al.*, 2004). However, no studies have directly examined the possible protective role of exercise-induced production of HSPs in human skeletal muscle against damage *in vivo*. This area therefore warrants further investigation and is discussed in section 8.4

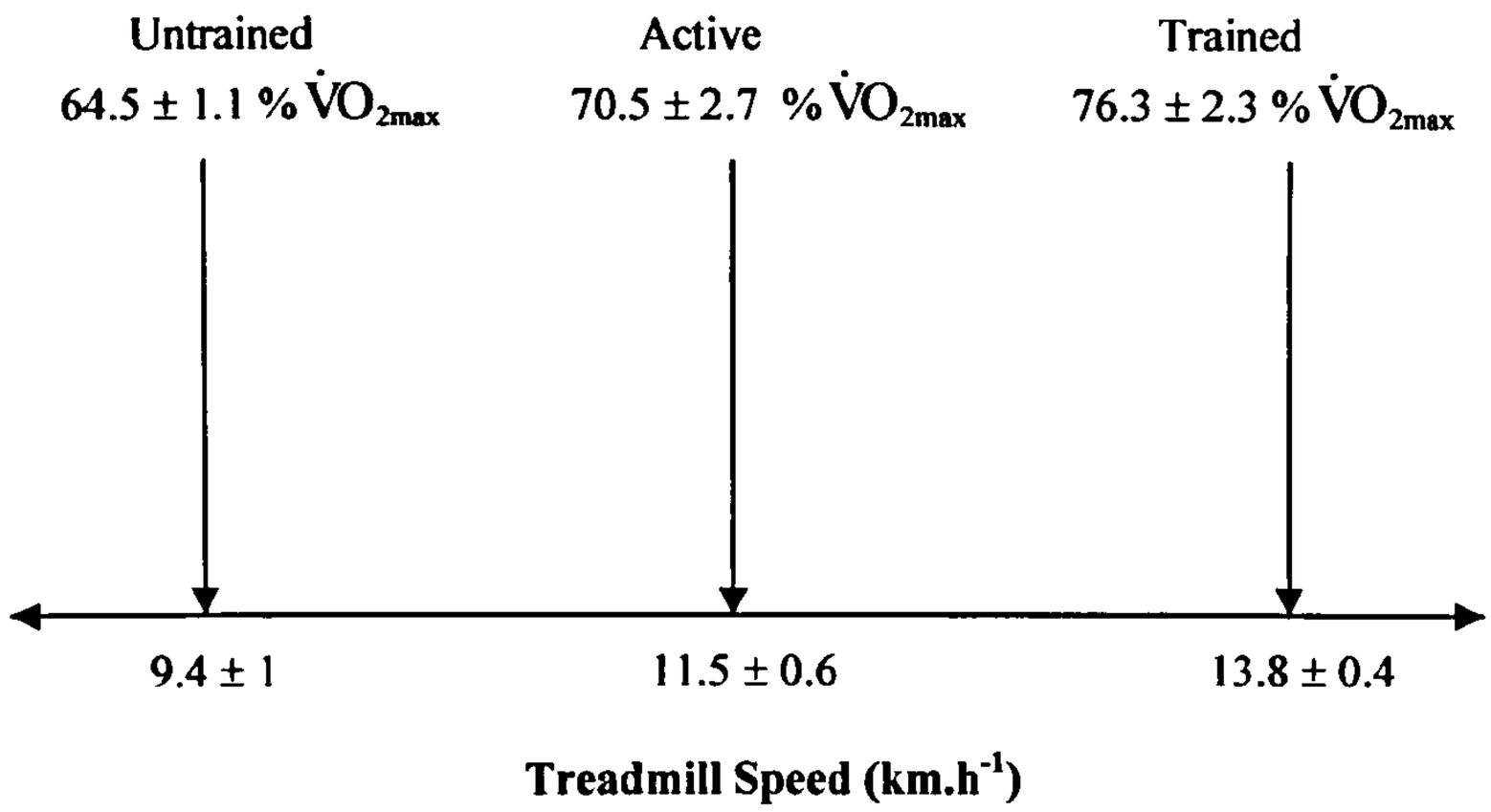
In addition to a training-induced adaptation of basal HSP status, the present thesis has also demonstrated that training status can influence the magnitude of the stress response to acute exercise. In Study 5, neither the trained or untrained subjects exhibited a stress response to the exercise protocol. This is in contrast to the active population examined in Study 3. The absence of a stress response in trained subjects confirmed the author's hypothesis and was attributed to the increase in baseline defence systems for these individuals and the customary nature of the exercise protocol. Indeed, whilst the exercise protocol for trained subjects (76 ± 2.3 % of $\dot{V}O_{2max}$) may have challenged less conditioned subjects, it was somewhat representative of daily training intensity for these individuals and is likely to have

failed to initiate a significant homeostatic challenge for these individuals. These data are in agreement with findings from rodent muscle in which trained rodents also exhibited a blunted stress response to customary exercise (Smolka *et al.*, 2000).

The absence of a stress response in untrained subjects was contrary to the author's hypothesis and was suggested to be due to the low intensity nature of the exercise protocol for this population. Despite being matched for lactate threshold, the protocol for untrained subjects equated to lower exercise intensities (range: 60 - 65% of $\dot{V}O_{2max}$) with treadmill speeds associated with brisk walking and gentle jogging pace. These data therefore led to a re-examination of the rationale for the chosen exercise intensity and the most appropriate way for which to match exercise intensity between subjects when examining the stress response to acute exercise. It was initially decided to match exercise intensity between groups for lactate threshold so as to elicit similar relative circulatory and metabolic responses between subjects (Baldwin *et al.*, 2000). However, when integrating the findings from Study 3 and Study 5, it would appear that the exercise-induced production of HSPs is determined by a 'critical exercise intensity threshold' that is more dependent upon the attainment of an absolute work load. This critical threshold appears further complicated by recruitment of a significant proportion of total muscle fibres, training status, baseline defences and novelty of the exercise stress.

Had the exercise intensity of both groups been matched according to $\dot{V}O_{2max}$ (e.g. 70%), it would have normalised the absolute workload between subjects, which may have led to similar total fibre recruitment patterns between groups (see Figure 8.1). This may be of particular relevance for induction of the stress response since the exercise-induced production of HSPs in rodent muscle following acute treadmill running is related to muscle fibre recruitment (Milne and Noble, 2002). In order to perform at this higher workload, the less conditioned muscle of untrained subjects would subsequently experience an increase in total fibre recruitment and a greater metabolic strain (e.g. increased ROS production, carbohydrate utilisation etc). In this way, the less conditioned muscle of untrained subjects may thereby need to exhibit a larger production of HSPs than trained muscle so as to combat the increased homeostatic disruption to protein structure and function.

A



B

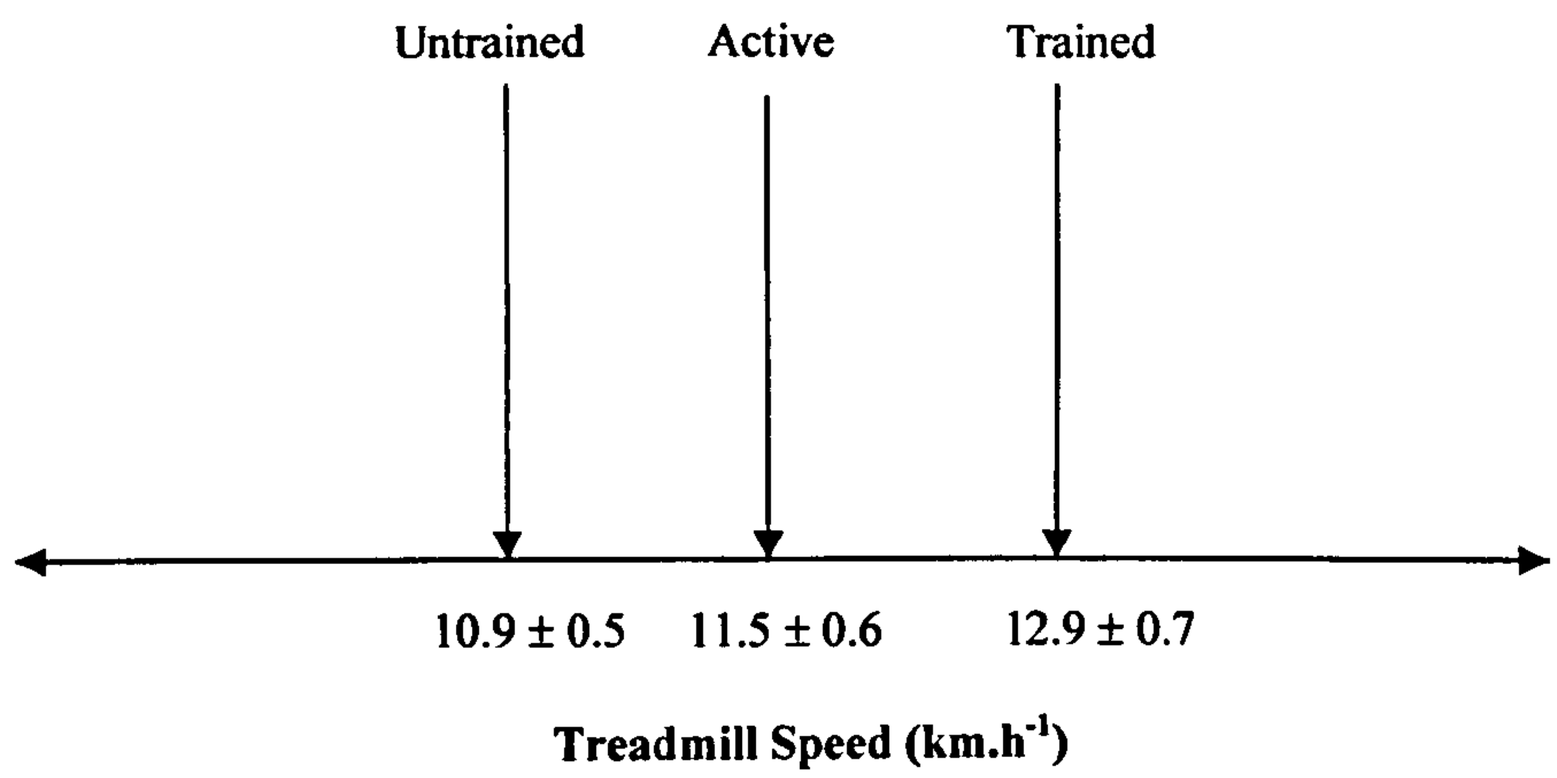


Figure 8.1 – (A) Comparison of treadmill speeds (i.e. absolute workloads) between the untrained, active and trained subjects when exercising at the lactate threshold. When exercising at these intensities, only the active subjects exhibited a stress following exercise. It is suggested that the absence of a stress response in trained subjects was due to an increase in baseline systems and the customary nature of the exercise protocol. The absence of a stress response in untrained subjects may be related to failure of the exercise protocol to elicit the critical threshold intensity that is required to induce HSPs. (B) Comparison of treadmill speeds (i.e. absolute work loads) between subjects if the exercise protocol was performed at 70% $\dot{V}O_{2max}$ as opposed to lactate threshold. In this situation, the absolute workload is normalised between groups. Here, it is suggested that untrained subjects would display a clear stress response to exercise because of the increased total fibre recruitment and metabolic demands that would be required to exercise at this higher absolute workload. When comparing the stress response between different populations, it is therefore recommended that exercise intensity be matched for percentage of $\dot{V}O_{2max}$ as the attainment of an absolute workload appears critical for induction of HSPs.

Taken together, the exercise related data presented in the present thesis therefore suggest that the exercise-induced production of HSPs is dependent upon a critical threshold (i.e. absolute workload) which is further complicated by training status and novelty of the stress. Indeed, as alluded to earlier when discussing the absence of a stress response in trained subjects, it appears that the exercise protocol must be relatively novel so as to overwhelm or challenge baseline defences. A proposed model depicting this possible exercise intensity dependent relationship is displayed in Figure 8.2.

This model suggests that specific populations will have a critical threshold exercise intensity beyond which exercise induces significant increases in muscle HSP content. It is proposed that the magnitude of the exercise-induced stress response for a given absolute workload will depend on the prior training induced basal levels of HSPs. This area is discussed further in section 8.4 as a recommendation for future research. The possible factors contributing to this critical threshold intensity are shown in Figure 8.3.

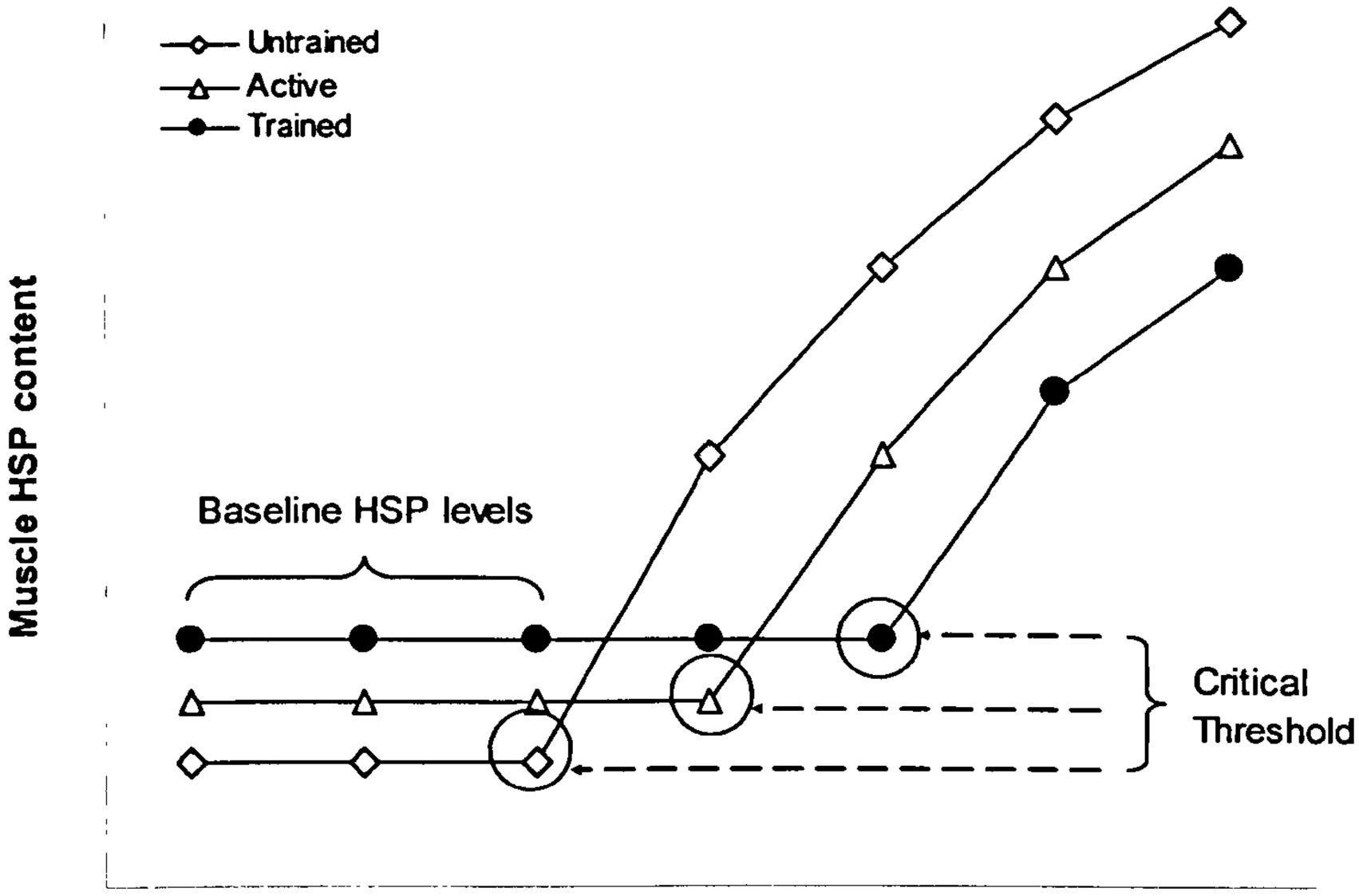


Figure 8.2 – Proposed inter-relationship between exercise intensity, training status and magnitude of the exercise-induced production of HSPs. This model suggests that specific subject populations will up-regulate HSP content when the attainment of the critical threshold has been achieved. The model also suggests that the magnitude of the stress response for a given absolute workload is influenced by the training induced adaptations of basal HSP levels.

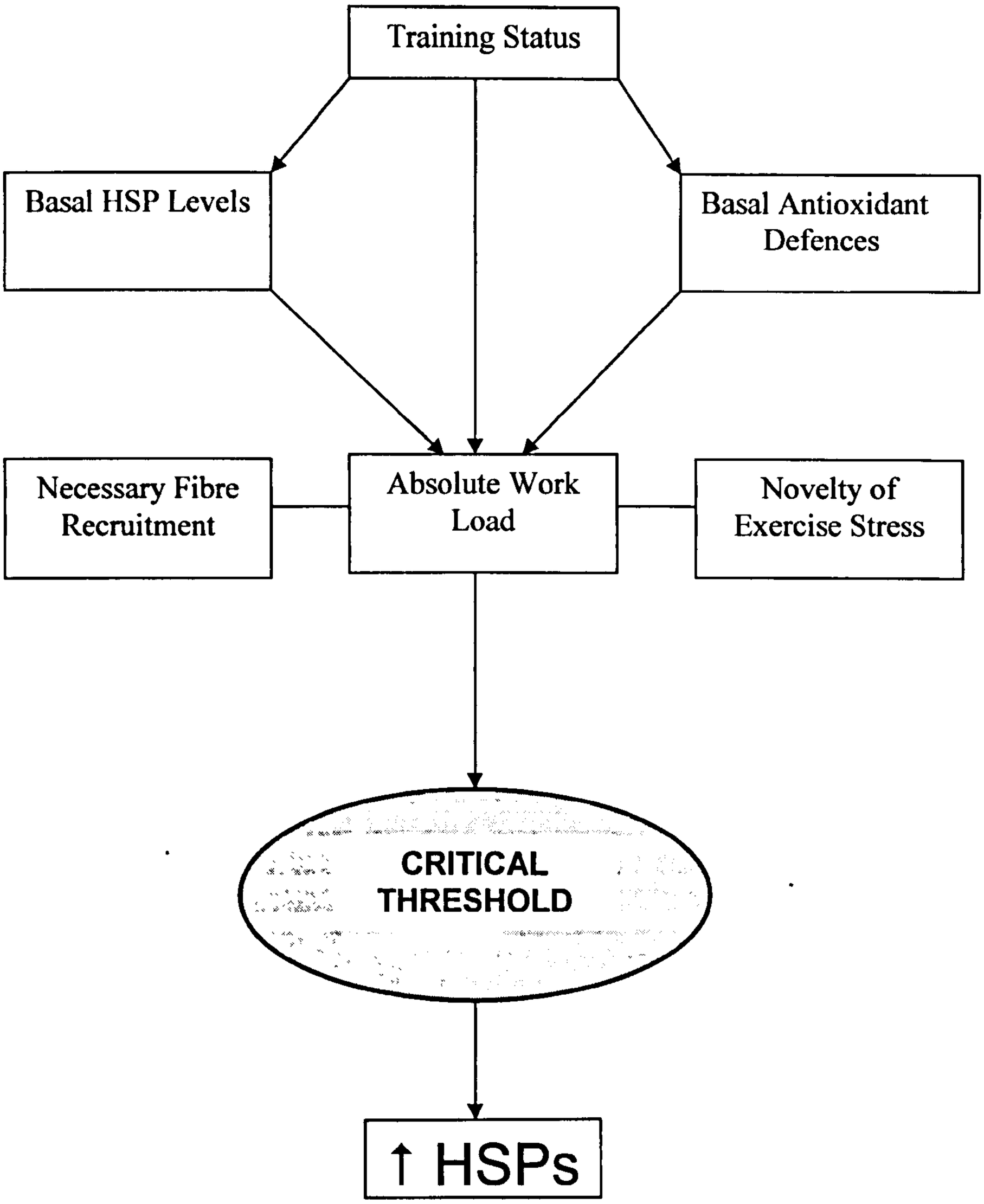


Figure 8.3 – Possible factors contributing to the attainment of a 'critical threshold' that is necessary for induction of HSPs following acute aerobic exercise. The model suggests that training status (and the accompanying basal levels of HSPs and antioxidant defences) will determine the absolute workload that is necessary to attain the critical threshold. This workload should be sufficient to activate a significant proportion of total muscle fibres and must also be relatively novel so as to overwhelm baseline defence systems.

8.3 CONCLUSIONS

The aims and objectives outlined in Chapter 1 have been achieved by completion of the experimental studies detailed in Chapters 4 – 7. These studies have provided novel data for the literature and have significantly advanced our understanding of the exercise-induced stress response of male human skeletal muscle.

The following conclusions can now be made:

1. Skeletal muscle of young active male subjects responds to the stress of an acute bout of non-damaging running exercise (performed at an intensity corresponding to the lactate threshold) via a differential up-regulation of HSPs. This response is variable between subjects although peak responses are typically observed at 48 h post-exercise.

2. Elevations in core and muscle temperature to similar levels as that typically occurring during moderate intensity non-damaging running exercise do not induce increases in HSP content in the skeletal muscle of active young males.

3. Trained subjects display a selective up-regulation of several HSPs and antioxidant defences in rested skeletal muscle than compared to untrained individuals.

4. Trained and untrained subjects do not display any increase in muscle HSP content following an acute bout of non-damaging running exercise performed at an intensity corresponding to the lactate threshold.

8.4 RECOMMENDATIONS FOR FUTURE RESEARCH

There are several potential areas of future research which have emerged from the data presented in this thesis, namely those of the impact of exercise intensity and training status on HSP production and the role of ROS in activating the exercise-induced stress response. The precise biological significance of the exercise-induced production of HSPs, particularly in relation to their potential cytoprotective properties, also warrants further study. In the following section, the reader is provided with a brief outline of potential studies addressing these issues. A proposed experimental design for such studies is initially discussed and is shown in Figure 8.4.

8.4.1 The temporal relationship between signalling pathways, HSF1 activation, HSP gene expression and increased HSP content

The time-course of exercise-induced HSP production is now relatively well established. Based on the present thesis and related data (Puntschart *et al.*, 1996; Khassaf *et al.*, 2001; Walsh *et al.*, 2001), increases in muscle HSP content are unlikely to occur until 48 h post-exercise. However, the temporal relationship between possible signalling pathways, HSF1 activation, increases in HSP gene expression and increased muscle HSP levels remains to be evaluated in human skeletal muscle. It is therefore recommended that additional biopsy samples be obtained immediately post- and up to 2 h post-exercise in all future studies in order to assess these parameters (see Figure 8.4). Venous blood samples should also be obtained at the time of biopsy sampling as they may provide an indirect indicator of the magnitude of exercise-induced muscle damage (e.g. CK levels) and possible signalling pathways (e.g. ROS production in muscle as assessed by electron spin resonance, ESR; Close *et al.*, 2004). This design will equate to a total of 5 biopsies and thus should conform to standard ethical guidelines. In addition to western blot analyses for specific proteins, it is recommended that proteomic analysis also be performed so as to provide an assessment of global changes in protein expression in differing populations both at rest and following exercise.

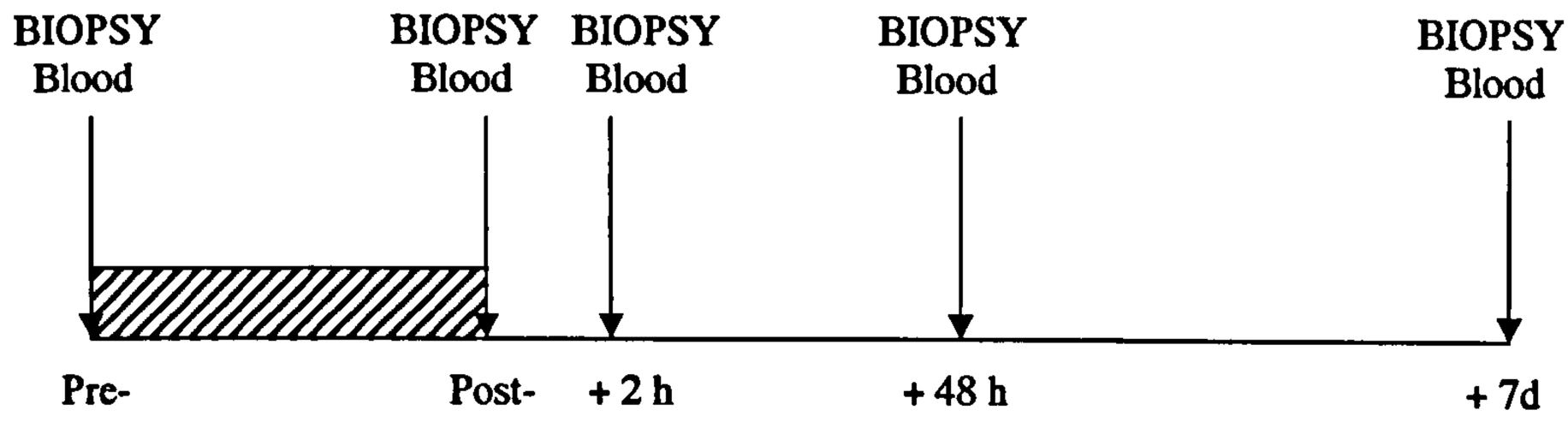


Figure 8.4 – Proposed experimental design for future research. Shaded area represents acute bout of exercise, the precise characteristics of which remain to be determined. It is recommended that additional biopsies be obtained immediately post- and at 2 h post-exercise so as to provide an assessment of possible signalling pathways, HSF1 activation and HSP gene expression.

8.4.2 The impact of exercise intensity and training status on HSP production: the 'critical threshold' hypothesis

It is now apparent that exercise intensity is likely to influence the magnitude of the stress response in a manner that is dependent on individual training status. In a randomised order, it is therefore suggested that untrained, active and trained young male subjects perform an acute bout of exercise at differing intensities of step-wise increments e.g. 60, 70 and 80% of $\dot{V}O_{2max}$. These intensities of exercise should also be quantified with respect to the lactate threshold. The precise duration of the exercise protocol would be prescribed from pilot work on the basis of subject completion rates. These data will test the proposed critical threshold hypothesis outlined in Figure 8.2 and 8.3. This work would also yield important data on the impact of exercise intensity on ROS production and free radical mediated signalling and damage, a relationship which has yet to be substantiated in human skeletal muscle. The impact of varying exercise protocols (e.g. continuous vs intermittent) on the magnitude of HSP production also warrant further study as they may be of importance significance for exercise programs designed at improving both health and performance.

8.4.3 The role of ROS in activation of the stress response

Antioxidant supplementation studies have demonstrated a role of ROS in signalling the exercise-induced stress response of human skeletal muscle (Khassaf *et al.*, 2003; Jackson *et al.*, 2004; Fischer *et al.*, 2006). These studies are limited, however, in their failure to provide direct assessment of ROS production or provide a multi-assay approach to demonstrate indirect indicators of ROS formation. The precise mechanisms by which ROS mediate the stress response and by which antioxidants are inhibitory, therefore remain to be defined. In a double blind cross-over design, it is suggested that active young male subjects perform an acute bout of exercise (the precise characteristics of which remain to be determined) before and after a period of antioxidant supplementation. It is hypothesised that antioxidant supplementation will lead to a reduction in muscle ROS production (as assessed by ESR) thereby leading to a reduced oxidation of protein thiols which, in turn, will result in lack of HSF1 activation and no transcriptional activity of the HSP genes.

8.4.4 The role of HSPs in reducing the extent of exercise-induced muscle damage

The precise biological significance and physiological role of the exercise-induced production of HSPs remain to be elucidated. An increased muscle HSP content following an acute exercise stress likely functions to restore homeostasis, facilitate increased protein turnover / remodelling and to provide cytoprotection against further exercise stresses. In keeping with this hypothesis, cells demonstrating elevated levels of HSPs following mild or non-damaging stresses display an enhanced protection against subsequent periods of normally damaging stresses (Garramone *et al.*, 1994; Lepore *et al.*, 2000; McArdle *et al.*, 1997; F. McArdle *et al.*, 2004; Maglara *et al.*, 2003; Suzuki *et al.*, 2000). It has therefore been suggested that increased levels of HSPs that occur during repetitive exercise (i.e. exercise training) function to maintain homeostasis during the stress of everyday activity and to offer increased protection against muscle damage induced by further exercise stresses. However, evidence for a protective role of increased HSP levels against contraction-induced damage of human skeletal muscle *in vivo* is currently lacking. In a matched subjects design containing 3

groups, it is therefore suggested that one group of active young male subjects perform an acute bout of non-damaging exercise known to induce an increase in muscle HSP content. At the time of peak HSP content, subjects will then perform a damaging exercise protocol to assess the impact of increased HSP content on susceptibility to exercise-induced muscle damage. In order to provide causative evidence for a protective role of HSPs *per se*, a control group would also perform the damaging exercise protocol having performed no prior non-damaging exercise. Furthermore, the remaining group would also perform the pre-conditioning non-damaging exercise protocol after a period of antioxidant supplementation known to inhibit the exercise-induced production of HSPs. It is hypothesised that enhanced protection against damage will only be evident in the non-supplemented group who performed the prior non-damaging exercise stress.

8.4.5 Wider implications

The ability of exercise to induce increases in muscle HSP content and their potential cytoprotective properties may have wider implications as therapeutic agents in a number of pathologies. In this regard, future research should also examine the impact of age and gender on the exercise-induced stress response of skeletal muscle. Such data may enhance our understanding of the mechanisms by which exercise can provide protection to cells and tissues against various stressors. The area of HSPs, muscle and exercise remains a potentially exciting and fruitful research area.

Chapter 9

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Appendices

Publications and Communications

Appendix I

Publication I

Morton, J.P., Atkinson, G., MacLaren, D.P.M., Cable, N.T., Gilbert, G., McArdle, A., Broome, C. and Drust, B.

Reliability of maximal muscle force and voluntary activation as markers of exercise-induced muscle damage

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ORIGINAL ARTICLE

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Reliability of maximal muscle force and voluntary activation as markers of exercise-induced muscle damage

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Abstract The loss of the ability of skeletal muscle to generate force is one of the most appropriate and valid means to quantify muscle damage. Routine measurements of maximal muscle force, however, include many potential sources of error, the most important of which may be a possible lack of central drive to the muscles. The aim of the present study was to determine the reliability of maximal isometric quadriceps muscle force and voluntary activation over a timescale that is typically employed to examine the aetiology of exercise-induced muscle damage. We also attempted to characterise the reliability of several twitch interpolation variables including the size of the interpolated twitch and the state (i.e. unpotentiated vs potentiated) and size of the resting twitch. Over a 7-day period, eight healthy active males performed repeated maximal voluntary isometric contractions (MVC) of the quadriceps (baseline and 2 h, 6 h, 24 h, 48 h, 72 h and 7 days post). Systematic variations in maximal muscle force, voluntary activation, interpolated twitch, unpotentiated twitch and potentiated twitch were not statistically significant ($P > 0.05$) and 95% repeatability coefficients of ±76.03 N, ±4.42%, ± 8.44 N, ±25.92 N and ±43.58 N were observed, respectively. These data indicate that young healthy well-familiarised male subjects can reproduce their perceived maximal efforts both within and between days where activation levels of >90% are routinely achieved. Providing activation remains within these limits in the 7 days following an acute bout of exercise, the researcher would be 95% certain that exercise-induced muscle damage is present in individual subjects (taken from similar subject populations) if MVC force falls outside these limits.

Keywords Electrical stimulation · Muscle contraction · Isometric force · Familiarisation

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Introduction

The loss of the ability of skeletal muscle to generate force is considered one of the most appropriate and valid means to quantify exercise-induced muscle damage (Faulkner et al. 1993; Faulkner and Brooks 1997; McArdle and Jackson 1999; Warren et al. 1999). The most frequently assessed variable is that of a maximal voluntary contraction (MVC), which is typically defined as the maximal muscle force that a highly motivated subject is able to produce voluntarily under particular contractile conditions. The reductions in MVC force associated with damage persist over the entire timescale of the progression of the degenerative and regenerative process i.e. until the muscle returns to its pre-damage condition (Warren et al. 1999). This cannot be said of other commonly used markers such as the release of intramuscular proteins into the circulation, soreness and histopathology.

The assessment of skeletal muscle function as a marker of damage necessitates reliable measures of maximal force. Routine measurements of maximal muscle force have many potential sources of error including a lack of central drive to the muscles (Merton 1954; Rutherford et al. 1986; Bulow et al. 1993). The degree of central activation is rarely taken into consideration when assessing maximal muscle force in the research area of exercise-induced muscle damage (Warren et al. 1999). This makes it difficult to ascertain if any post-exercise reductions in force are actually representative of structural damage to the muscle or simply a reduction in voluntary drive. It has therefore been

recommended that the twitch interpolation technique has to be routinely employed in muscle damage studies so as to provide an objective assessment of central activation pre- and post-exercise (McArdle and Jackson 1999; Byrne et al. 2004; Millet and Lepers 2004).

The twitch interpolation technique involves the delivery of one or more electrical impulses (via nerve, percutaneous or cortical stimulation) to the active muscle during an MVC. The failure of an interpolated twitch to produce an increment in force during an MVC suggests maximal voluntary activation and maximal force. The presence of a visible twitch, on the other hand, demonstrates that the force produced by the muscle is less than maximal and suggests that either not all motoneurons have been recruited or that they are discharging at sub-optimal firing rates (Todd et al. 2004). Using simple ratios of the size of the superimposed twitch with respect to the size of the same stimulus evoked in a resting muscle (Merton 1954; Gandevia 2001) or to the level of voluntary torque (Kent-Braun and Le Blanc 1996; Knight and Kamen 2001), the level of voluntary activation can be readily quantified as an interpolated twitch ratio (ITT) or central activation ratio (CAR), respectively.

Although several researchers have examined the reliability of the twitch interpolation technique for the assessment of maximal quadriceps muscle force and voluntary activation (Behm et al. 1996, 2001; Norregaard et al. 1997; Oskeui et al. 2003; Todd et al. 2004), to the authors' knowledge no researchers have formally tested the reliability of these variables across a timescale (i.e hours to days) so that the temporal pattern of skeletal muscle damage in response to acute exercise can be characterised. Such temporal evaluations of reliability estimates are of particular importance given that the reliability of any assessment should always be established with respect to its intended use or 'analytical goal' (Atkinson and Nevill 1998).

The aim of the present study is therefore to examine the reliability of measurements of maximal isometric quadriceps muscle force and voluntary activation (using the twitch interpolation technique) across a timescale that is typically employed to examine the aetiology of exercise-induced muscle damage. It is intended to provide a baseline data set highlighting the variability of maximal force and activation in a healthy young male population free of any exercise-induced muscle damage. Given that estimates of voluntary activation can be affected by the size of the interpolated twitch and the state (potentiated vs unpotentiated) and size of the resting twitch, we also attempted to characterise the variability associated with each of these variables.

Methods

Subjects

Eight healthy active males volunteered to participate in the study (mean ± SD: age 21 ± 1 years; mass 84 ± 8 kg; height 1.8 ± 0.02 m). All subjects gave written informed consent to participate after details and procedures of the study had been fully explained. Subjects refrained from exercise throughout the study and from alcohol and caffeine intake for at least 24 h prior to any of the testing sessions. All subjects had no history of neurological disease or musculoskeletal abnormality and none were under any special pharmacological treatment during the course of the study. The study was approved by the Ethics Committee of Liverpool John Moores University.

Familiarisation

Subjects underwent extensive familiarisation prior to participating in the reliability study. During such sessions, the subjects were introduced to and familiarised with the procedure of performing isometric MVCs of the quadriceps (4 s duration) with and without twitch interpolation. In the initial session, subjects practiced performing MVCs without twitch interpolation so as to get accustomed to the concept of achieving and maintaining voluntary force. This session was also utilised to obtain maximal current tolerance and establish the supra-maximal current amplitude for superimposition during an MVC. Whilst remaining at rest, the amperage of a 250 V square wave pulse (100 μs, 1 Hz) was progressively increased until the point beyond which further

Table 1 MVC force and voluntary activation of three representative subjects during the familiarisation period. These subjects needed varying degrees of familiarisation before being considered as eligible to participate in formal reliability testing. Data presented for sessions 2–6 are representative of those trials involving electrical stimulation only and correspond to the peak values observed in that session

		Familiarisation session					
		1	2	3	4	5	6
Subject 1	MVC force (N)	720.6	745.5	778.9	802.1	811.3	815.6
	Voluntary activation (%)	–	87.6	89.1	93.1	96.1	95.9
Subject 2	MVC force (N)	950.4	975.3	966.8			
	Voluntary activation (%)	–	97.8	97.2			
Subject 3	MVC force (N)	729	788.3	838.7	845.1		
	Voluntary activation (%)	–	91.4	93.5	94.1		

increases in intensity caused no further increase in resting twitch force (Newman et al. 2003). In subsequent sessions, subjects alternated between performing MVCs with and without twitch interpolation so that approximately three to four trials of each were performed in each session. This approach of alternating between MVCs with and without stimulation was undertaken because many subjects, at this early stage, performed weaker contractions when they were expecting stimulation (presumably because of apprehension by the prospect receiving noxious stimuli) compared to when they were not expecting stimulation. Familiarisation sessions were completed until subjects' MVC force and voluntary activation demonstrated a plateau effect between sessions. This level of initial consistency was usually achieved within three to seven sessions (mean ± SD: 5 ± 2 sessions) after which subjects were then considered eligible to participate in formal reliability testing. Familiarisation data from three representative subjects who needed varying degrees of familiarisation are displayed in Table 1.

Design

Between 1 days and 3 days after familiarisation, subjects completed seven test sessions over a period of 7 days. Each session involved five trials of 100% MVC during which twitches were superimposed. The first three of these sessions occurred in 1 day (immediately pre- 1 h of a hypothetical exercise bout and 2 h and 6 h post-hypothetical exercise) with the remaining sessions occurring at 24 h, 48 h, 72 h and 7 days after the hypothetical exercise bout. A schematic of the experimental procedure is shown in Fig. 1.

Maximal isometric quadriceps force

The isometric force of the quadriceps of the subject's dominant leg was measured with the subjects sitting upright in a testing chair. Subjects were seated with the trunk vertical with a 90° flexion in the hip and knee. To prevent extraneous body movements, velcro straps were applied tightly across the thorax and proximal thigh. Quadriceps muscle force was measured from the ankle where the attachment was connected to a strain gauge by a metal force transducer (previously calibrated with known weights). After a standard warm-up period, subjects performed five trials of 100% MVC (4 s duration) during which supra-maximal twitches were superimposed (see below). A 3-min rest period was included between each trial in an attempt to eliminate the effects of fatigue (Newman et al. 2003). Subjects were given standardised strong verbal encouragement during each trial and real-time visual feedback of their performance was provided via the projection of the computer display onto a large screen placed in front of the subject. The force signal was A/D converted with a sampling frequency of 1,000 Hz. Data were acquired for 8 s and analysed with a commercially designed software programme (AcqKnowledge III, Biopac Systems, Massachusetts).

Twitch interpolation

The quadriceps were electrically stimulated using two moistened surface electrodes (Chattanooga, USA, 7×12.7 cm) which were positioned proximally over the vastus lateralis and distally over the vastus medialis. Skin preparation for each electrode included shaving and light abrasion of the skin followed by cleansing with an isopropyl alcohol swab. A permanent marking pen was used to outline the position of each electrode so as to minimise electrode placement variability from session to session (Keogh et al. 1999).

Eight single square wave electrical impulses (100 μs) were delivered during the 8 s sampling period. Each impulse was computer driven and was delivered at 250 V (Digimeter DS7, Hertfordshire, UK). Two impulses were delivered before and after the contraction. The peak forces of the pre- and post-contraction twitches were averaged so as to allow comparison of resting twitch amplitudes in an unpotentiated and potentiated condition, respectively (Oskouei et al. 2003). The remaining four impulses were delivered during the contraction and tested the maximality of each MVC. The amplitude of supra-maximal superimposed current was that identified for each subject in familiarisation sessions and corresponded to 10% above the level required to evoke a resting muscle twitch of maximal amplitude (Todd et al. 2004). An example MVC including the timing of each twitch is provided in Fig. 2.

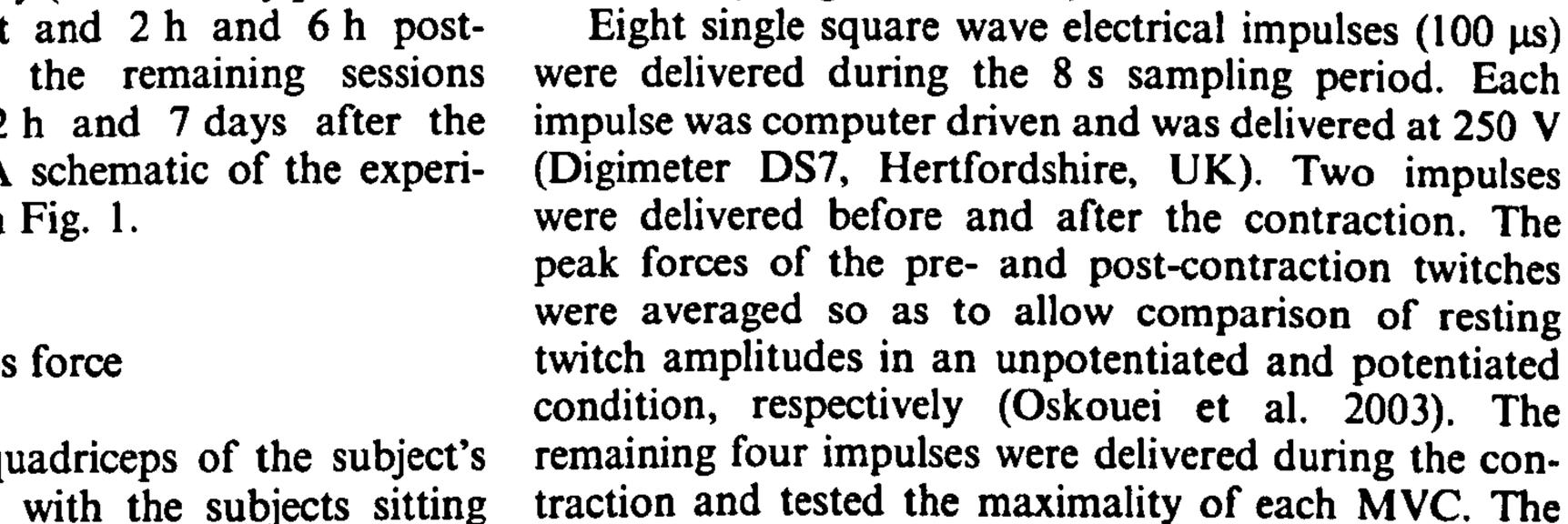

Fig. 1 Schematic illustration of the experimental design. *Shaded area* represents 1 h of a hypothetical exercise bout. Each subject performed five MVCs with twitch interpolation (*) in each testing session

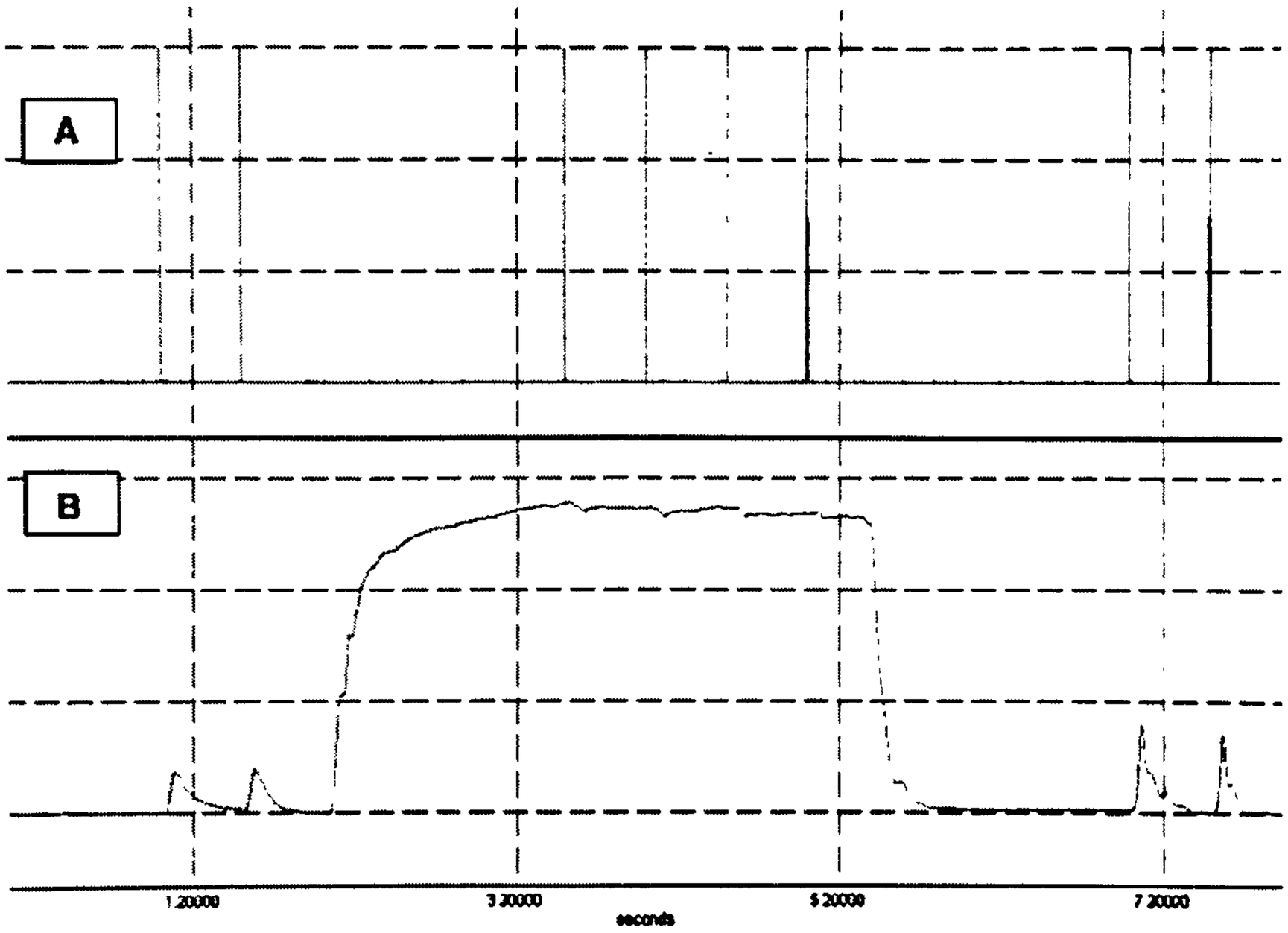

Fig. 2 Typical MVC with twitches superimposed. *Top panel* (**A**) represents timing of superimposed twitches. *Bottom panel* (**B**) represents the subject's force-trace

Activation levels

Voluntary activation was calculated according to an interpolated twitch ratio (as advocated by Merton 1954) whereby the size of the interpolated twitch was expressed as a ratio of the amplitude evoked by the same stimulus delivered to a relaxed potentiated muscle (see Eq. 1). The average force during a 100 ms period before the application of each stimulus during the contraction and the maximal force during a 100 ms period after each stimulus that was applied were recorded (Graven-Neilsen et al. 2002). The highest 100 ms mean pre-stimulus force (taken as MVC force) and the resulting maximal post-stimulus force were subsequently used for calculation of the size of the interpolated twitch whereby interpolated twitch size was calculated by subtraction of the mean pre-stimulus force from the maximal post-stimulus force.

$$\text{Voluntary activation} = \left[1 - \left\{\frac{\text{size of interpolated twitch}}{\text{size of resting twitch}}\right\}\right] \times 100 \quad (1)$$

Statistical and data analysis

Mean MVC force, voluntary activation, interpolated twitch size and resting twitch size (unpotentiated and potentiated) were recorded for each subject for each session. All data are presented as means (±SD) with probability values of <0.05 assumed to indicate statistical significance.

Any systematic differences in MVC force, voluntary activation, interpolated and resting twitch amplitudes across sessions were assessed using a repeated measures general linear model (GLM). Ninety-five percent confidence intervals for the largest mean differences between time points were also calculated.

Random errors between test times were explored for heteroscedasticity (Nevill and Atkinson 1997) using the predicted vs residuals plot from the GLM. Within-subject standard deviations were calculated (in both absolute terms and as coefficients of variance) as were the 95% "repeatability coefficients" (Bland and Altman 1999) from $1.96x\sqrt{2xMSE}$ where MSE = mean square error term from the GLM output (Nevill and Atkinson 1998).

A two-way repeated measures GLM was used to examine for systematic differences in potentiated and unpotentiated resting twitches over time.

Results

Reliability of MVC force and voluntary activation

The variability in MVC force and voluntary activation across sessions is presented in Fig. 3. There was no significant change in MVC force across the seven sessions ($F_{6,42}=1.789$, $P=0.125$). The average MVC force during the 7 days was 934±95.9 N with individual values ranging from 835.29 N to 1151.85 N. The largest mean difference between any test sessions was 39.8 N (95% confidence intervals: 18.6–61.0 N). The 95% repeatability coefficient for MVC force was ± 76.03 N. This statistic estimates that in individual healthy young

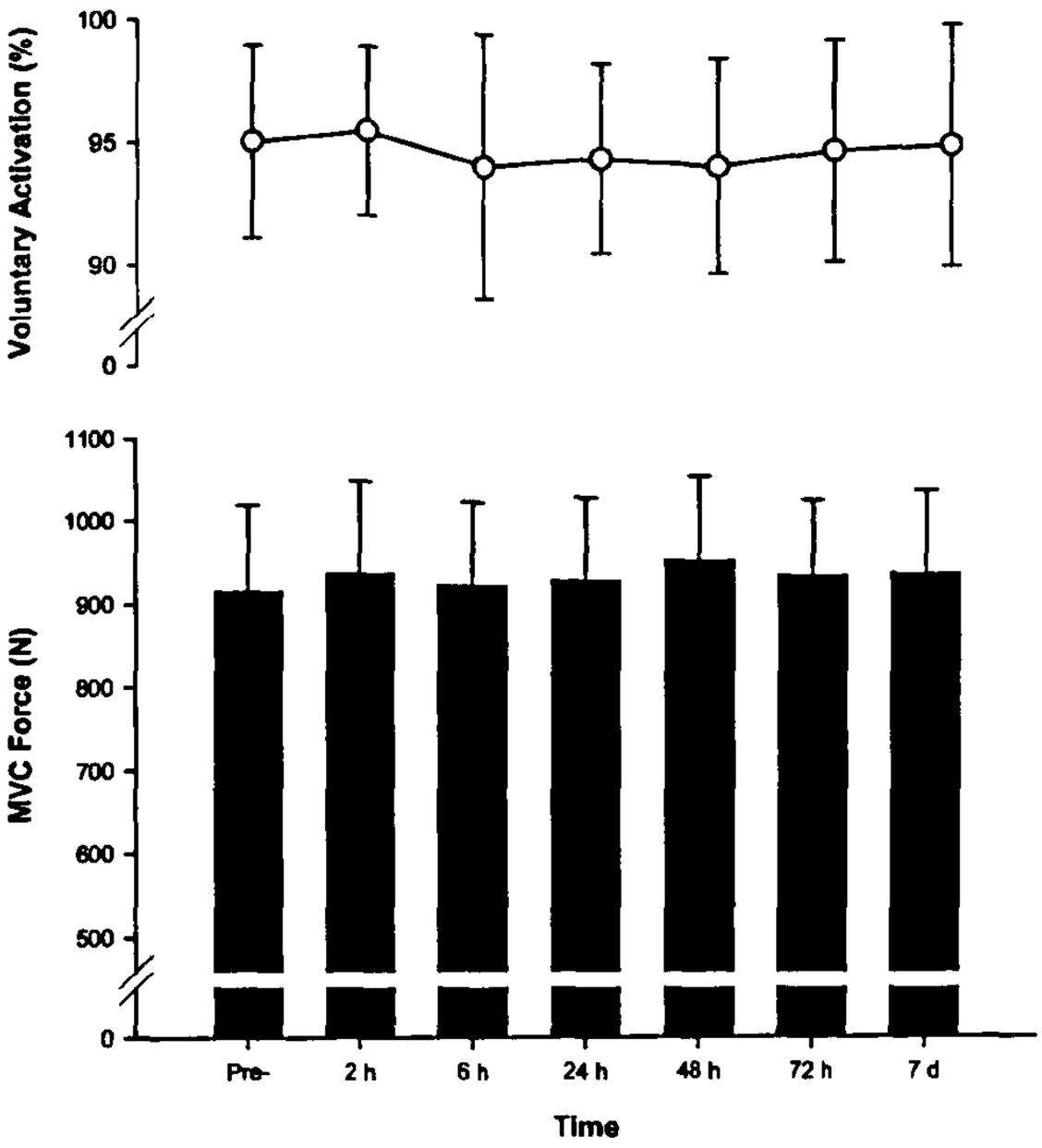

Fig. 3 MVC force and voluntary activation during the 7-day testing period

males who display no symptoms of exercise-induced muscle damage, 95% of the differences in MVC force measured in repeated trials across 7 days will not exceed or be less than 76.03 N.

In accordance with MVC force, voluntary activation did also not significantly change during the seven sessions ($F_{6,42}=0.928$, $P=0.485$). The average voluntary activation during the 7 days was 94.6± 4.1% with individual values ranging from 86.0% to 97.4%. The largest mean difference between any test sessions was 1.4% (95% confidence intervals: 1.2–4.1%). The 95% repeatability coefficient for voluntary activation throughout the seven sessions was ±4.42%.

Coefficients of variation (CVs) for each subject's MVC force and voluntary activation percentage over the 7-day period are presented in Table 2. Individual CVs for MVC force and voluntary activation ranged from 2.98–6.52 and 1.62–5.27%, respectively.

Variability of interpolated twitches

The variability in the size of the interpolated twitches across sessions is presented in Fig. 4. There was no significant change in the interpolated twitch force over the 7 days ($F_{6,\ 42}=2.186$, $P=0.063$). The average size of the interpolated twitch during the 7 days was 9.92 ± 7.54 N with individual values ranging from 3.19 N–26.34 N. The largest mean difference between any test sessions was 4.6 N (95% confidence intervals: 0.1–9.2 N). The 95% repeatability coefficient for the interpolated twitch size was ±8.44 N.

Table 2 Coefficients of variation (%) for each subject's MVC force and estimates of voluntary activation during the 7-day period

Subject	MVC force	Voluntary activation
1	2.98	1.77
2	4.41	4.32
3	4.72	3.19
4	4.68	4.48
5	6.52	1.62
6	3.98	5.27
7	3.68	3.01
8	3.41	3.36
Mean	4.31	3.38
SD	1.09	1.28

Variability in resting twitches

The variability in resting twitch size across sessions is presented in Fig. 5. There was no significant change in either unpotentiated or potentiated resting twitches over the 7 days ($F_{6,42}=1.594$, $P=0.173$). As expected, there was a significant difference between potentiated and unpotentiated resting twitches ($F_{1,42}=136.42$, $P<0.001$). The average size of the unpotentiated twitch was 111.64 ± 13.69 with individual values ranging from 97.54 N to 139.78 N. The average size of the potentiated twitch was 181.72 ± 27.33 N with individual values ranging from 160.3 N to 244.62 N. Potentiated and unpotentiated twitches corresponded to 19.58 ± 3.17 and 12.03 ± 1.66% of MVC force, respectively. The largest mean difference between any test sessions for unpotentiated and potentiated twitches was 11.0 N (95% confidence intervals: 3.6–18.5 N) and 17.7 N respectively (95% confidence intervals: 4.4–39.9 N). The 95% repeatability coefficients for unpotentiated and potentiated resting twitches were ±25.92 N and ±43.58 N, respectively.

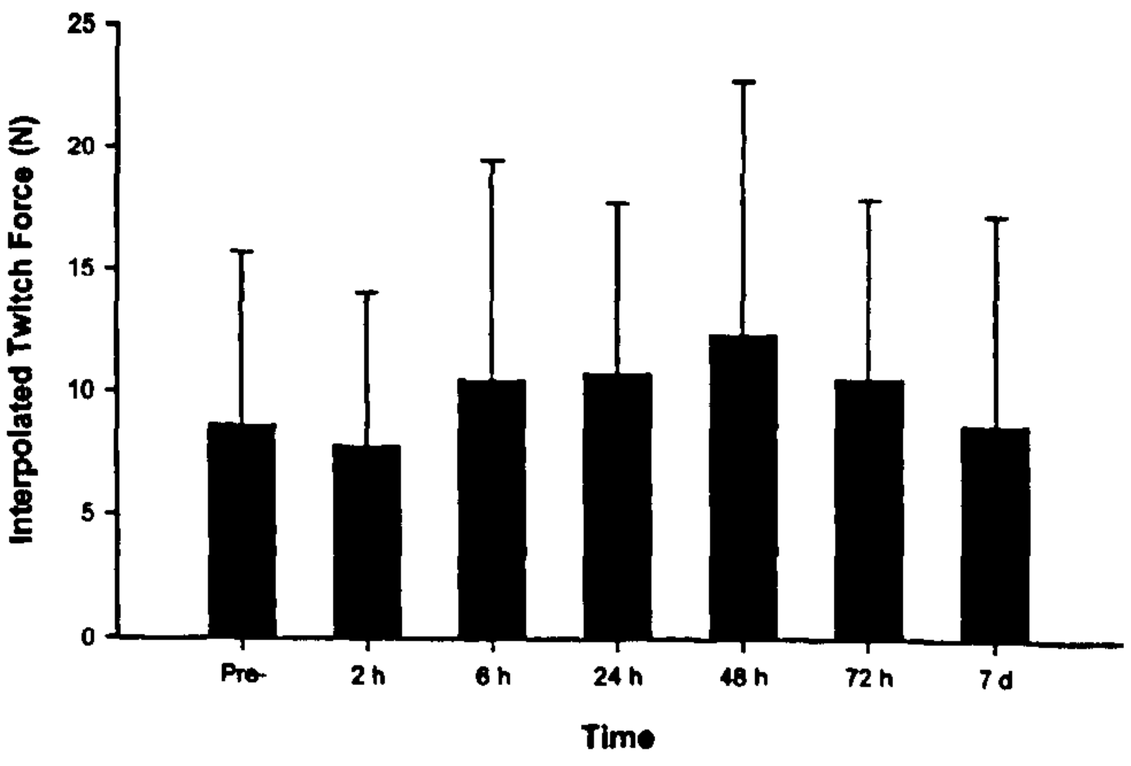

Fig. 4 Variations in the interpolated twitch force during the 7-day testing period

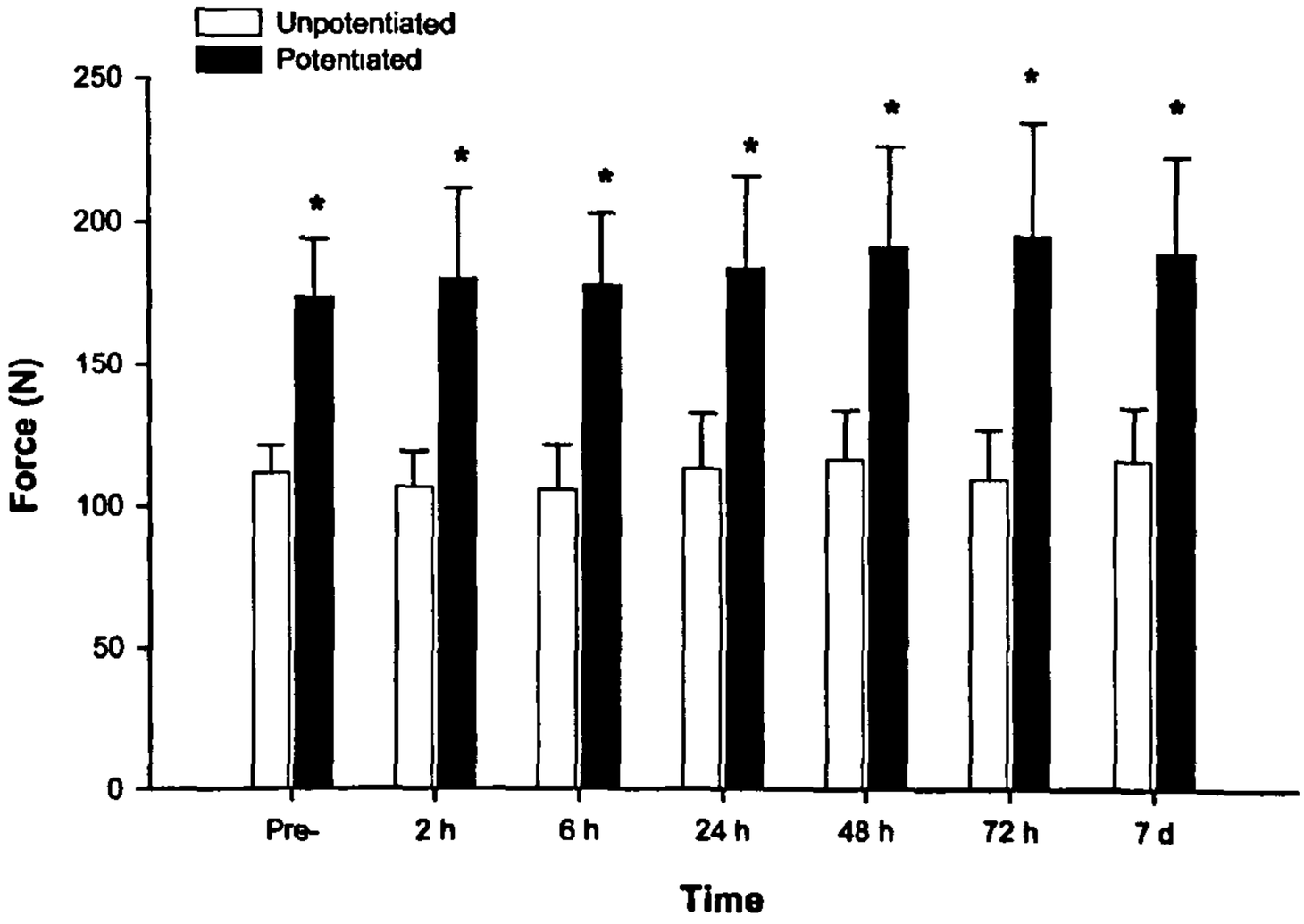

Fig. 5 Effects of potentiation on resting twitch force across sessions. * denotes significant difference between potentiated and unpotentiated twitches, $P < 0.001$

Discussion

The novel aspect of the present study was the evaluation of reliability estimates of maximal muscle force, voluntary activation and twitch related variables over a timescale that is typically employed to examine the aetiology of exercise-induced muscle damage. We have quantified reliability using the 95% repeatability coefficient (Bland and Altman 1999) and have thus provided a baseline data set highlighting the variability associated with maximal quadriceps muscle force and voluntary activation in a healthy young male population free of any muscle damage. Our data has characterised a level of measurement error deemed 'acceptable' for how these variables are to be assessed in future research.

There was no evidence of any systematic bias in maximal quadriceps force or voluntary activation during the 7-day testing period. These data suggest that any initial 'learning' or 'training' effect was alleviated during familiarisation. These results indicate that well-familiarised healthy young male subjects are therefore capable of reproducing their perceived maximal efforts both within day and between days. Indeed, individual CVs for MVC force and voluntary activation over the 7-day period were extremely low and ranged from 2.98–6.52% and 1.62–5.27%, respectively.

The 95% repeatability coefficient for the repeated measurements of MVC force and voluntary activation were ±76.03 N and ±4.42%, respectively. Providing activation remains within these limits in the days following an acute bout of exercise, the researcher would be 95% certain that muscle damage is present in a healthy young male subject if MVC force falls outside these limits. We have since conducted research in our laboratory using a downhill running protocol as a damaging intervention which resulted in force reductions of approximately 200–300 N at 24 and 48 h post-exercise. Individual changes in voluntary activation did not exceed the limit outlined above.

In the present study, the average voluntary activation percentage during the 7 days was 94.6% with individual values ranging from 86.0% to 97.4%. Only in 21 of the total 280 maximal attempts was the interpolated twitch completely occluded (i.e. full activation achieved). These values compare well with previous studies where voluntary activation levels of 85–95% have typically been reported for the quadriceps (Suter et al. 1996; Shield and Zhou 2004). It is now well documented that in comparison with other muscles, voluntary activation of the quadriceps is substantially harder to achieve (Behm et al. 2002). This finding is likely explained by the size principle of motor recruitment (Henneman et al. 1974) whereby the predominantly fast twitch knee extensors (Gollnick et al. 1973) are more difficult to voluntarily activate given the higher recruitment threshold of fast twitch motor neurons.

In accordance with the recent views of Todd et al. (2004), we are also of the opinion that several important experimental factors underlie the highly reliable nature of our data. These include the extensive familiarisation period that each subject undertook prior to participating in formal reliability testing, the use of real-time visual feedback of each subject's force production, the provision of subjects with strong verbal encouragement during each contraction and also the use of a quiet and private room to conduct all testing sessions. All of these factors can contribute to subjects' motivation levels which are of paramount importance in achieving reliable and valid maximal efforts.

Oskouei et al. (2003) recently reported random variations in the interpolated twitch force when subjects performed ten repeated maximal contractions of the quadriceps. These authors suggested that small variations in voluntary force between contractions could not account for the variations in interpolated twitch force.

Oskouei et al. (2003) described their findings as 'disappointing' as maximal effort contractions are the most frequently used contractions in the clinical setting. We have extended the previous findings by highlighting the variability in interpolated twitch force over a much larger sampling period and have quantified this random error using the 95% repeatability coefficient where a value of 8.44 N was calculated. In agreement with Oskouei et al. (2003), we also have no insight as to what causes the variations in superimposed twitch force. It is apparent that random variations exist that are therefore inherent of the twitch interpolation technique particularly for maximal or near maximal (>90%) contractions of the quadriceps.

As expected, there was a significant difference in potentiated and unpotentiated twitch forces. Although neither twitch force significantly changed during the 7 days, potentiated twitches appeared to be somewhat more variable than unpotentiated twitches (95% repeatability coefficients, 43.58 N vs 25.92 N). The enhanced variability associated with potentiated twitches is most likely related to timing of twitches. The potentiation effect, although apparent for minutes (Bulow et al. 1993), decreases very quickly after relaxation and can change by as much as up to 50% in the first 10 s after deactivation. In the present study, potentiated twitches were delivered at approximately 1.5 and 2 s after the subject had relaxed. However, this time interval was by no means exact given that individuals displayed different rates of relaxation that also varied both within session and between sessions. It is therefore recommended that special attention be given to the timing of potentiated twitches so as to ensure that each twitch is delivered as precisely as possible after deactivation.

In summary, it is concluded that young healthy well-familiarised male subjects can perform reliable and reproducible measures of maximal quadriceps force and voluntary activation across a timescale that is typically used to examine the temporal pattern of exercise-induced muscle damage. The variability associated with voluntary activation and maximal force (in individual patients who are free of any symptoms of exercise-induced muscle damage) can be estimated in healthy young males using the 95% repeatability coefficient where values of 4.42% and 76.03 N were observed respectively. Such a population can routinely achieve activation levels of >90% although full voluntary activation is rarely achieved in either single or repeated trials. The twitch interpolation technique should be frequently employed in muscle damage research so as to ensure that any observed post-exercise reductions in voluntary force are not simply a reflection of a reduced central drive. We strongly recommended that each individual laboratory conduct a similar temporal and statistical evaluation of reliability estimates that is specific to the particular population under consideration (i.e. age and gender specific) prior to beginning any related experimental research.

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Appendix II

Publication II

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Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise

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Morton, James P., Don P. M. MacLaren, Nigel T. Cable, Thomas Bongers, Richard D. Griffiths, Iain T. Campbell, Louise Evans, Anna Kayani, Anne McArdle, and Barry Drust. Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise. *J Appl Physiol* 101: 176–182, 2006. First published March 24, 2006; doi:10.1152/japplphysiol.00046.2006.—The exercise-induced expression of heat shock proteins (HSPs) in rodent models is relatively well defined. In contrast, comparable data from human studies are limited and the exercise-induced stress response of human skeletal muscle is far from understood. This study has characterized the time course and magnitude of the HSP response in the skeletal muscles of a healthy active, but untrained, young male population following a running exercise protocol. Eight subjects performed 45 min of treadmill running at a speed corresponding to their lactate threshold (11.7 ± 0.5 km/h; 69.8 ± 4.8% maximum O_2 uptake). Muscle biopsies were obtained from the vastus lateralis muscle immediately before and at 24 h, 48 h, 72 h, and 7 days postexercise. Exercise induced a significant ($P < 0.05$) but variable increase in HSP70, heat shock cognate (HSC) 70, and HSP60 expression with peak increases (typically occurring at 48 h postexercise) to 210, 170, and 139% of preexercise levels, respectively. In contrast, exercise did not induce a significant increase in either HSP27, αB-crystallin, SOD 2 (MnSOD) protein content, or the activity of SOD and catalase. When examining baseline protein levels, HSC70, HSP27, and αB-crystallin appeared consistently expressed between subjects, whereas HSP70 and MnSOD displayed marked individual variation of up to 3- and 1.5-fold, respectively. These data are the first to define the time course and extent of HSP production in human skeletal muscle following a moderately demanding and nondamaging running exercise protocol. Data demonstrate a differential effect of aerobic exercise on specific HSPs.

chaperones; oxidative stress; hyperthermia; muscle temperature

ONE OF THE MOST PROMINENT cellular responses to stress is a rapid change in gene expression to yield a family of highly conserved proteins known as heat shock proteins (HSPs) (19, 20, 32, 48). In the unstressed cell, HSPs function as molecular chaperones associating with newly synthesized polypeptides to ensure correct folding, function, and location (48). An increased content of HSPs following stress functions to restore cellular homeostasis, facilitate successful repair from injury, and protect the cell against further insults (19, 26, 28, 29).

The stress of both acute (13, 21, 27, 31, 37, 40) and chronic exercise (12, 30, 35, 39) has been consistently shown to induce increases in HSP content in the skeletal muscle of various animal species. The stress response in rodent models is now relatively well defined and is typically observed several hours postexercise (25). Skeletal muscle also adapts to increased contractile activity via an upregulation of the antioxidant defense network (14). The adaptation of such defense systems offers a potential mechanism for the increased tolerance to exercise and protection from contraction-induced damage associated with exercise training.

Acute exercise also stimulates increased HSP production in human skeletal muscle. An increase in HSP70 content has been observed in the vastus lateralis following acute one-legged cycling (17, 18) and exhaustive knee extensor exercise (11) and in the biceps brachii following damaging contractions (42–44). An increased content of HSP27 and αB-crystallin has also been observed in the vastus lateralis following downhill running (8). Two studies have investigated the heat shock response (limited to HSP70) of human skeletal muscle following running exercise protocols (36, 47). These authors failed to detect an increase in HSP70 expression at 3 h (36) and 24 h (47) following periods of moderately demanding running exercise. Previous data from our laboratory (17), however, demonstrated that biopsy samples beyond 24 h postexercise are likely needed to detect exercise-induced changes in muscle HSP levels. A comprehensive time course study of the stress response following running exercise has yet to be performed. This is particularly important given the relevance of running to the general population (i.e., as a keep-fit activity) and in an array of sporting activities.

Despite these initial descriptions, the exercise-induced stress response of human skeletal muscle remains poorly characterized and understood. Interpretation of data from human studies is often limited to the response of one particular HSP family (most notably HSP70) and is complicated by the variations in timing of tissue sampling, differing subject characteristics (e.g., age, training status, gender, nutritional status), and the disparate exercise protocols utilized by investigators. This is particularly important where there is a damaging component to the exercise protocol (where damage is defined as gross necrosis and a significant reduction in the force-generating capability

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of the muscle) whereby phagocytic cells that migrate to the site of injury contain relatively high levels of HSPs (18). The use of a nondamaging exercise protocol, however, provides a more controlled methodological approach whereby the increased expression of HSPs is likely to have arisen from skeletal muscle cells rather than changes in phagocytic cell content.

The aim of the present study was to characterize the time course and pattern of response of the major HSP families in human skeletal muscle following acute, nondamaging treadmill running exercise. These include the HSP70 family [HSP70 and heat shock cognate (HSC) 70], mitochondrial HSP60, and two members of the small HSP family (HSP27 and αB-crystallin). Given recent evidence that baseline HSP levels display marked individual variation (17), we also examined the extent of individual variation of baseline HSPs and antioxidant protein levels.

MATERIALS AND METHODS

Development of a nondamaging exercise protocol. In a preliminary study, eight active male subjects [mean ± SD: age, 24 ± 3 yr; weight, 77 ± 6 kg; height, 1.79 ± 0.07 m; maximum O_2 uptake ($\dot{V}O_{2\,max}$), 56.3 ± 5 ml·kg^{-1}·min^{-1}; lactate threshold, 70.1 ± 3.4% $\dot{V}O_{2\,max}$] performed a 45-min treadmill running protocol at a speed corresponding to their lactate threshold (11.5 ± 0.9 km/h) on a motorized-driven treadmill (Woodway, Auf-Schrauben, Germany). Measurements of maximal quadriceps isometric force and voluntary activation were assessed immediately before and at 2 h, 6 h, 24 h, 48 h, 72 h, and 7 days postexercise, according to Morton et al. (33). Venous blood samples were also obtained at these time points and analyzed for serum creatine kinase activity, according to a modification of the spectrophotometric method of Jones et al. (15). Data deemed this protocol to be nondamaging in nature in that it resulted in no significant increases in serum creatine kinase levels (Fig. 1*A*) and no significant reductions in the force-generating capability of the quadriceps muscles (Fig. 1, *B* and *C*).

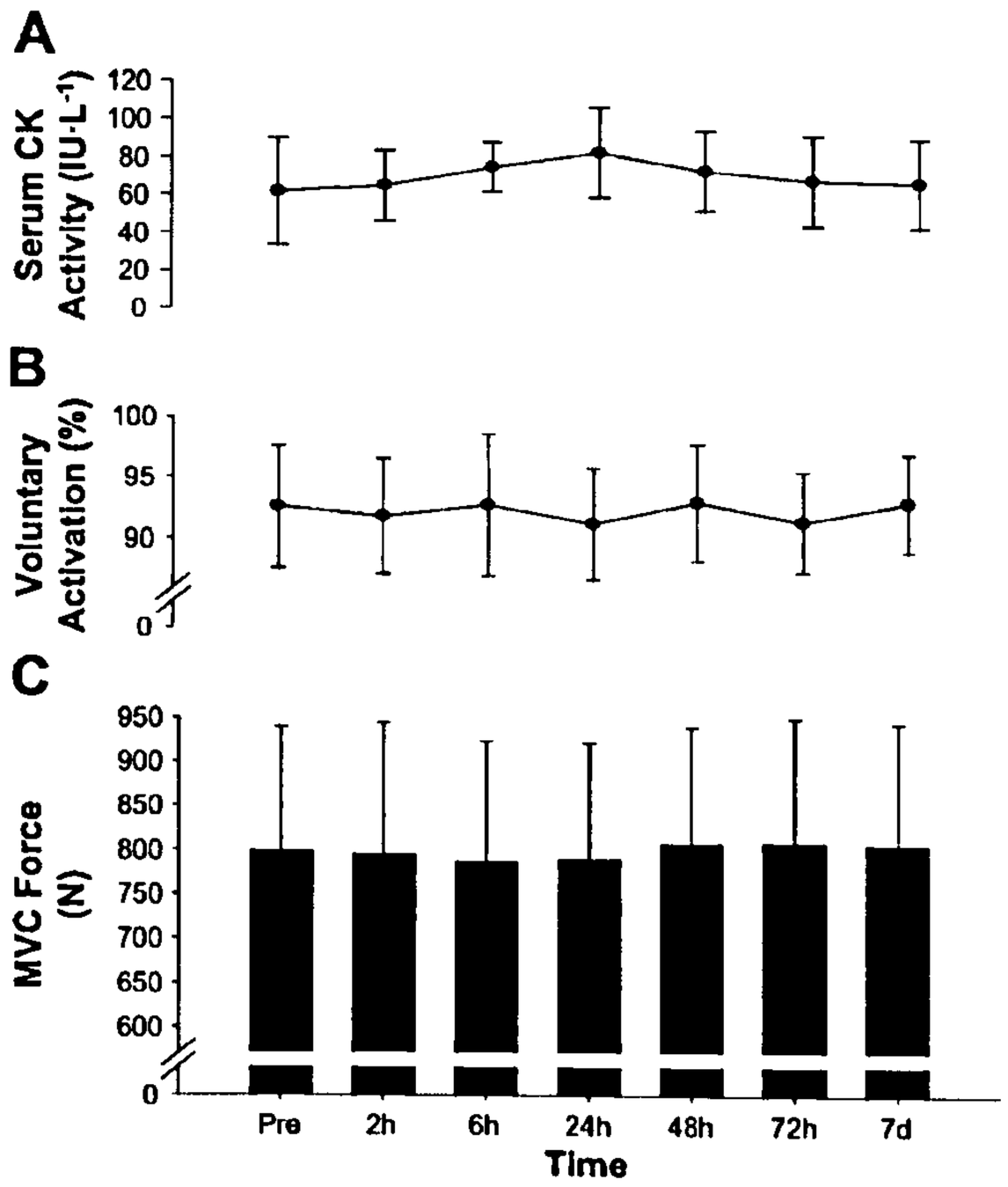

Fig. 1. Development of a nondamaging exercise protocol. *A*: serum creatine kinase (CK) activity. *B*: estimates of voluntary activation. *C*: maximal quadriceps isometric muscle force pre- (Pre) and postexercise. MVC, maximum voluntary contraction; d, day.

Subjects. Eight active but untrained men volunteered to participate in the study (mean ± SD: age, 24 ± 4 yr; weight, 78.9 ± 7.4 kg; height, 1.8 ± 0.05 m; $\dot{V}O_{2\,max}$, 54.9 ± 4 ml·kg^{-1}·min^{-1}; lactate threshold, 69.8 ± 4.8% $\dot{V}O_{2\,max}$). All subjects gave written, informed consent to participate after details and procedures of the study had been fully explained. Subjects refrained from any exercise (outside of the study) throughout the testing period and from alcohol and caffeine intake for at least 24 h before any of the testing sessions or muscle biopsy sampling. No subjects had a history of neurological disease or musculoskeletal abnormality, and none was under any pharmacological treatment during the course of the study. The study was approved by the Ethics Committee of Liverpool John Moores University.

Design and exercise protocol. Four to five days after having initially been assessed for $\dot{V}O_{2\,max}$ (6) and lactate threshold (4), subjects completed the 45-min nondamaging treadmill running protocol (outlined above) at a speed corresponding to their lactate threshold on a motorized-driven treadmill (Woodway). Muscle biopsies were obtained from the vastus lateralis immediately before the exercise protocol and at 24 h, 48 h, 72 h, and 7 days postexercise. This intensity of exercise (as opposed to percentage of $\dot{V}O_{2\,max}$) was chosen so as to normalize the exercise intensity between individuals with different aerobic capacities (2). This exercise intensity also compares well with similar intensities previously used in treadmill protocols examining the heat shock response to exercise (36, 47).

Ratings of perceived exertion (RPE) (3), thermal comfort scale (TCS) (45), and heart rate (Polar 610i, Kempele, Finland) were recorded at 5-min intervals throughout exercise. Oxygen uptake ($\dot{V}O_2$) was also measured (MetaLyzer 3B, Cortex Biophysic, Leipzig, Germany) for 5-min periods between 5–10, 20–25, and 35–40 min of exercise. Fingertip capillary blood samples were obtained at 15, 30, and 45 min and analyzed immediately in duplicate for whole blood lactate concentration (Lactate Pro, Arkray, Kyoto, Japan) to verify the intensity of exercise. Core temperature was measured using a rectal probe placed 10 cm beyond the anal sphincter (ELLAB) and was monitored continuously during exercise (CTF9004, ELLAB). Muscle temperature was measured in the vastus lateralis immediately pre- and postexercise using a needle thermistor (CTF9004, ELLAB) inserted to a depth of 3 cm (38). When the needle thermistor was removed, the injection site was cleaned with a sterile alcohol injection swab and covered with waterproof dressing. The ambient temperature of the laboratory during each exercise session was ~18 ± 0.8°C. Fluid intake was not permitted at any time during exercise.

Muscle biopsies. Muscle biopsies were taken from the vastus lateralis under local anesthesia (0.5% marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL). Biopsies on consecutive time points were taken from alternate legs, and samples obtained (~50 mg) were immediately frozen in liquid nitrogen and stored at −80°C for later analysis. Previous data have shown that the process of serial muscle biopsies per se is insufficient to induce the expression of HSPs (17), indicating that serial biopsies could be taken without inducing the production of stress proteins in the remaining tissue.

Biochemical procedures. Samples were homogenized in a 1% solution of SDS containing protease inhibitors (27). Each sample was centrifuged at 4°C, and the total protein content of the supernatant was measured using bicinchoninic acid (Sigma Chemical, Dorset, UK). Total protein was separated by SDS-PAGE using a 12% polyacrylamide gel and 4% stacking gel (National Diagnostics). Proteins were transferred onto a nitrocellulose membrane, as previously described (27). The muscle content of HSP70, HSC70, HSP60, HSP27, and αB-crystallin was analyzed using a panel of mouse monoclonal

Table 1. *Heart rate and ratings of perceived exertion and thermal comfort during the exercise protocol*

	Time, min					
	Preexercise	10	20	30	40	Postexercise
Heart rate, beats/min	76±6	164±6*	169±6*	174±5*	179±6*	179±6*
RPE	6	12±1*	13±1*	14±1*	16±2*	16±2*
TCS	5±1	6±1*	7±1*	7±1*	8±1*	8±1*

Values are means ± SD. RPE, ratings of perceived exertion; TCS, thermal comfort scale. *Significant difference from preexercise values, $P < 0.05$.

(HSP60 and HSP70), rat (HSC70), or rabbit polyclonal antibodies (HSP27, αB-crystallin, and MnSOD) (Stressgen, Victoria, Canada). Bands were visualized using an enhanced chemiluminescence detection system (Pierce) and Chemi-doc image capture system with Quantity One software (Bio-Rad). The content of HSPs was expressed as a percentage of the preexercise content for each subject. For analysis of antioxidant enzyme activity, samples were homogenized in 100 mM phosphate buffer (pH 7.0). Total SOD activity was measured according to the method of Crapo et al. (7). Catalase activity was measured by following the kinetic decomposition of hydrogen peroxide at 240 nm using a method derived from Claiborne (5).

Statistical analyses. Changes in exercise-related variables during the exercise protocol (i.e., heart rate, RPE/TCS, blood lactate, $\dot{V}o_2$, and core temperature) and changes in muscle HSP content and antioxidant enzyme activity following exercise were analyzed using repeated-measurements general linear models (GLM). Differences in muscle temperature between pre- and postexercise was assessed using a Student's *t*-test for paired samples. Where there was a significant main effect for time, paired *t*-tests with Bonferroni corrections were used for post hoc analysis. In a "summary of statistics" approach (1), Student's *t*-test for paired samples was also used to examine pre- to peak changes in HSP expression. Peak changes were taken as the time point at which subjects demonstrated their maximal change in muscle HSP content. Correlations between baseline protein levels were assessed using Pearson's correlation coefficient. All data are presented as means ± SD, with P values of <0.05 indicating statistical significance.

RESULTS

Physiological responses to the exercise protocol. The exercise protocol was performed at a running speed of 11.7 ± 0.5 km/h. Heart rate, RPE, and TCS during exercise are displayed in Table 1. All of these variables displayed a significant ($P < 0.05$) and progressive linear increase during exercise. $\dot{V}o_2$ and blood lactate values showed no significant change during exercise. Specifically, $\dot{V}o_2$ corresponded to 68.8 ± 4.8, 70.5 ± 3.8, and 69.5 ± 3.7% of $\dot{V}o_{2\,max}$ at 5–10, 20–25, and 35–40 min of exercise, respectively. Blood lactate values were 3 ± 0.4, 3.3 ± 0.6, and 3.5 ± 0.7 mmol/l after 15, 30, and 45 min of exercise, respectively. Core and muscle temperature changes during the exercise protocol are presented in Fig. 2. Exercise induced a significant rise ($P < 0.05$) in core temperature, increasing from 37.5 ± 0.2 at rest to 39.2 ± 0.3°C immediately postexercise. Muscle temperature also exhibited a significant increase ($P < 0.05$) during exercise, increasing from 36.2 ± 0.7 at rest to 40 ± 0.3°C immediately postexercise.

Changes in HSP content following exercise. Muscle HSP70 content showed a significant and variable increase following exercise (Fig. 3*A*). This response achieved significance ($P < 0.05$) at 48 h (179% of preexercise content) and 7 days postexercise (178% of preexercise content). When considering peak responses (Fig. 3*B*), which typically occurred at 48 h postexercise, HSP70 increased to 210 ± 70% of preexercise levels (range, 135–366%). The HSP70 response to exercise showed a marked individual variation in both magnitude and time course of the response. Representative Western blots highlighting an example of such individual variation are presented in Fig. 3, *C–E*.

The pattern of changes in muscle HSC70 content mirrored the response of HSP70, although this change was not statistically significant when assessed using a repeated-measures GLM (Fig. 4*A*). However, HSC70 increased significantly ($P < 0.05$) to 170 ± 75% of preexercise levels (range, 116–340%) at the time of peak expression, typically occurring at 48–72 h postexercise (Fig. 4*B*).

The muscle content of HSP60 showed no significant statistical change when assessed using a repeated-measures GLM (Fig. 5*A*). When examining peak responses (Fig. 5*B*), which again occurred at 48 and 72 h postexercise in individual

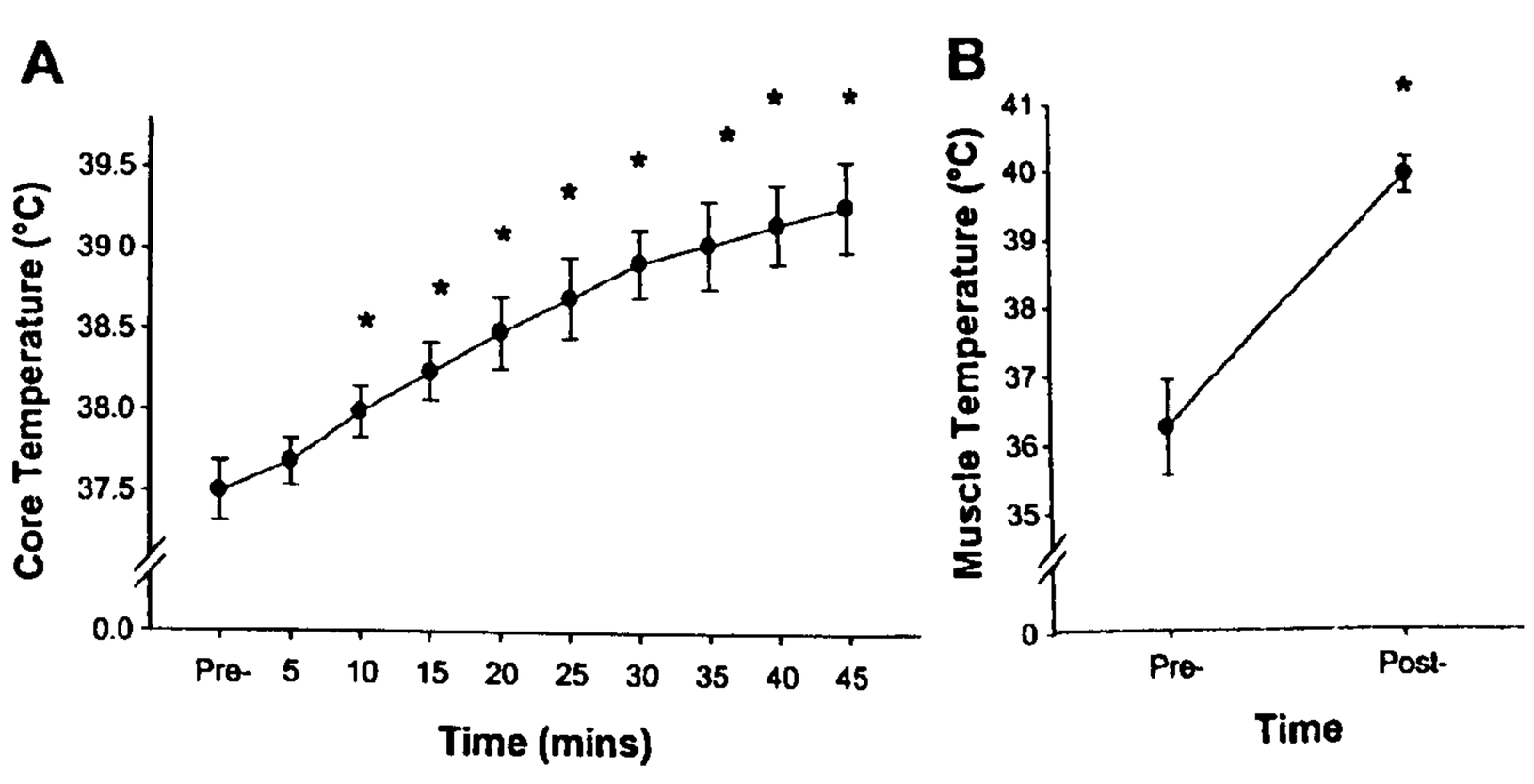

Fig. 2. *A*: core temperature during the exercise protocol. *B*: muscle temperature of the vastus lateralis immediately pre- and postexercise (Post). *Significant difference from preexercise values, $P < 0.05$.

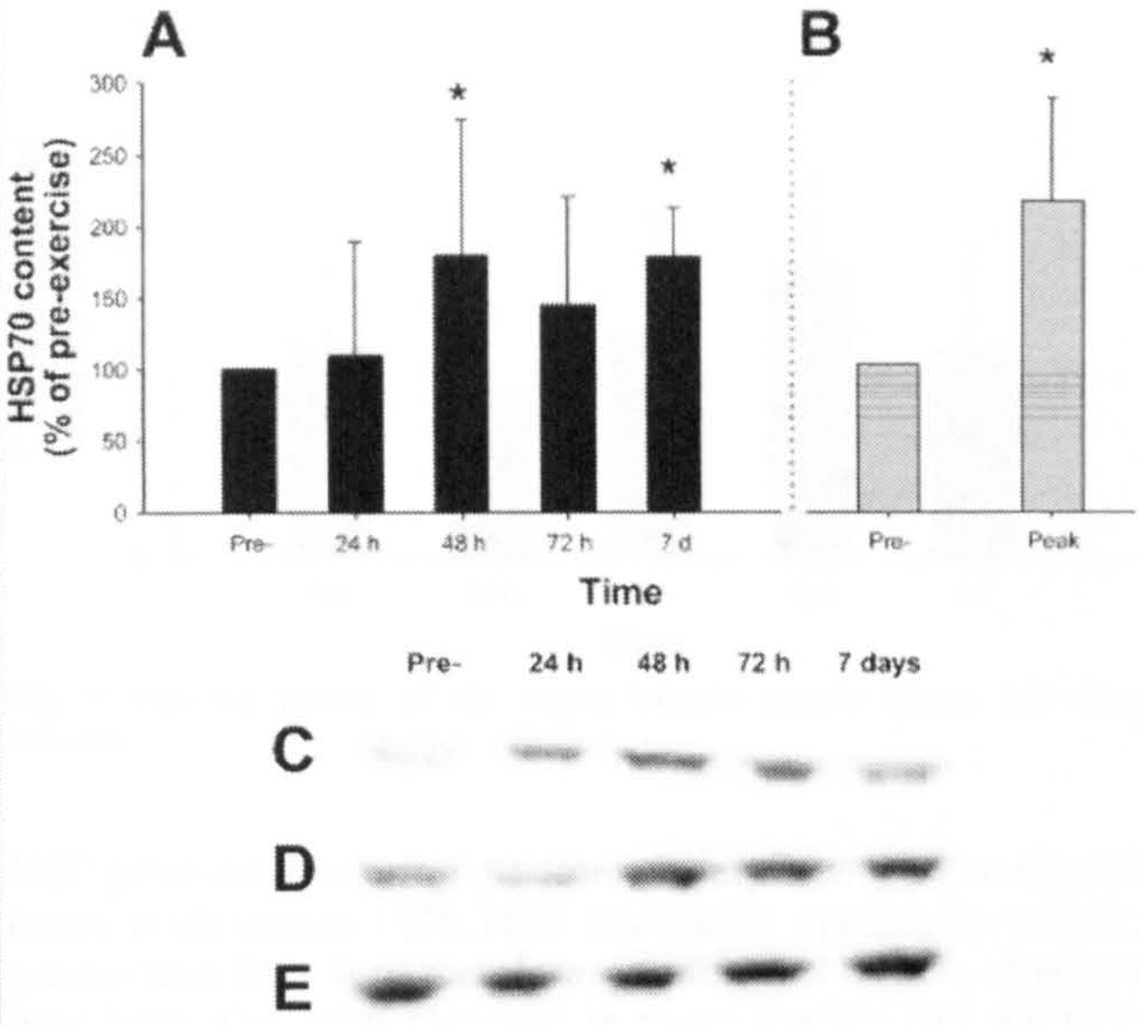

Fig. 3. *A*: changes in content of heat shock protein (HSP) 70 in the vastus lateralis before and after exercise. *B*: peak changes in HSP70 content. *Significant difference from preexercise values, $P < 0.05$. *C–E*: representative Western blots of individual subjects who showed varying HSP70 responses.

subjects, HSP60 increased significantly ($P < 0.05$) to 139 ± 23% of preexercise values (9–73%).

HSP27, αB-crystallin, and MnSOD protein content showed no significant change following exercise when assessed by either repeated-measures GLM or evaluating pre- to peak changes (data not shown).

Antioxidant enzyme activity. Muscle total SOD and catalase activity showed no significant change following exercise (Figs. 6 and 7, respectively).

Baseline HSP levels. A comparison of preexercise levels of muscle HSP content is shown in Fig. 8. Western blots from only seven subjects are presented due to insufficient availability from one subject. HSC70, HSP27, and αB-crystallin were constitutively expressed and showed little individual variation between subjects. In contrast, HSP70 and MnSOD expression displayed marked variation, exhibiting up to a 3- and 1.5-fold difference between subjects, respectively. Of all baseline proteins, correlations were only evident between HSP70 and MnSOD ($r = 0.81$).

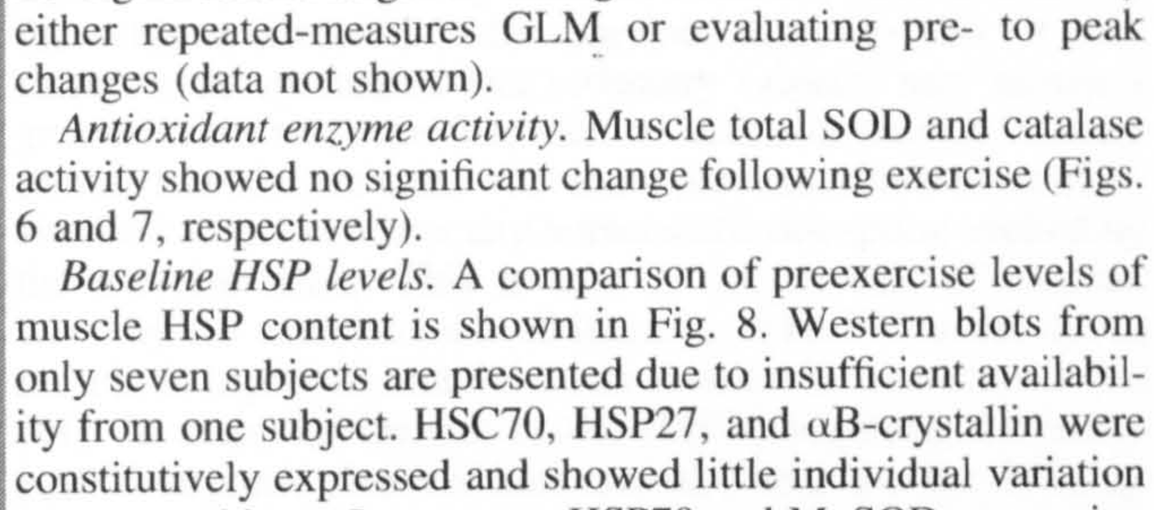

Fig. 4. *A*: changes in content of heat shock cognate 70 (HSC70) in the vastus lateralis before and after exercise. *B*: peak changes in HSC70 content. *Significant difference from preexercise values, $P < 0.05$.

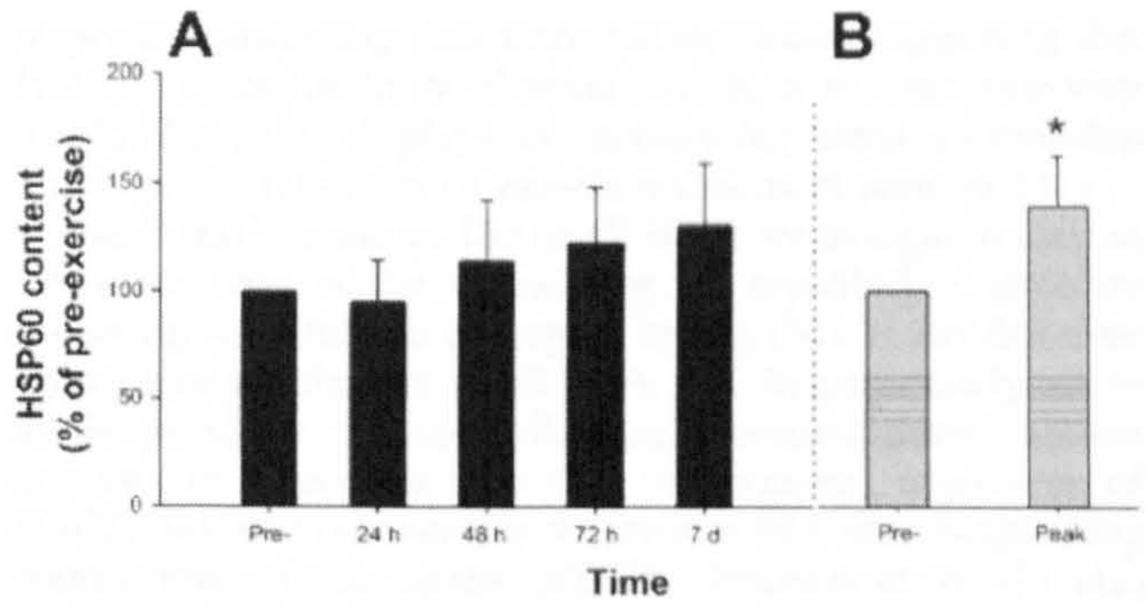

Fig. 5. *A*: changes in content of HSP60 in the vastus lateralis before and after exercise. *B*: peak changes in HSP60 content. *Significant difference from preexercise values, $P < 0.05$.

DISCUSSION

The present study has characterized the time course and magnitude of response of the major HSP families in the skeletal muscle of an active young male population following an acute bout of moderately demanding and nondamaging treadmill exercise. Our data provide novel data for the literature and have demonstrated that running exercise is a sufficient stimulus to upregulate the expression of several HSPs (most notably HSP70). Although examination of individual data reveals that 48 h postexercise appears to be an appropriate time point for which to detect maximal exercise-induced increases in HSP expression, we advocate the use of multiple postexercise biopsy samples in future studies. This is because the stress response appears highly variable between subjects in that some individuals may not display peak responses to 72 h or 7 days following exercise. Data also demonstrate a differential effect of aerobic exercise on specific HSPs.

HSP70 and HSP60 response. Muscle HSP70 and HSP60 content exhibited a peak significant increase of ~2-fold and 1.4-fold, respectively. The discrepancy between the present study and those who previously failed to observe a significant change in HSP70 content following running exercise may be related to restrictions of postexercise biopsy samples to within 1 day following exercise (36, 47). This is also a likely explanation for the absence of an increased HSP60 content immediately after 2-h cycling exercise (10). Whereas transcription of

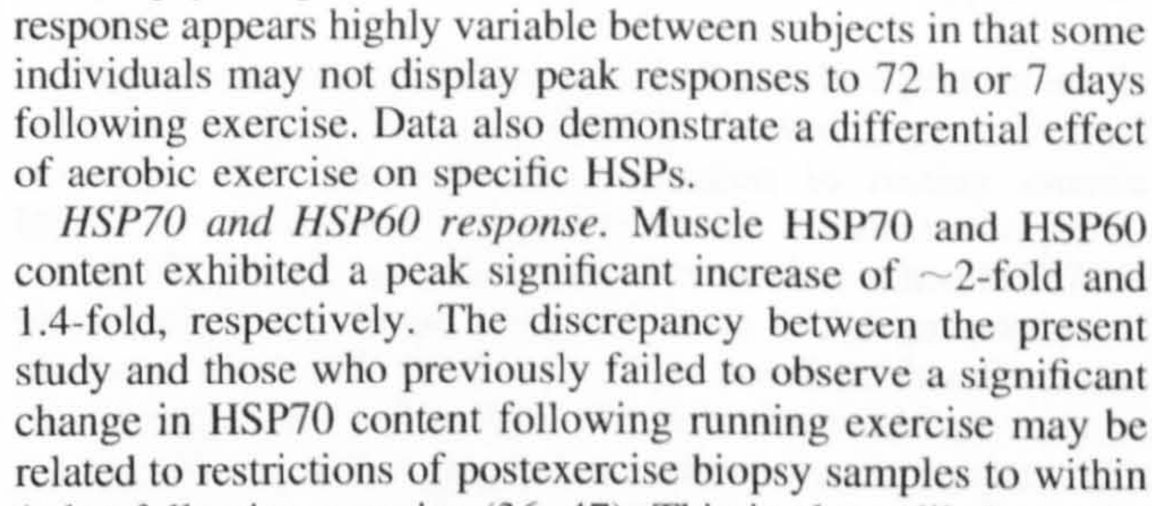

Fig. 6. Total superoxide dismutase (SOD) activity of the vastus lateralis muscle before and after exercise.

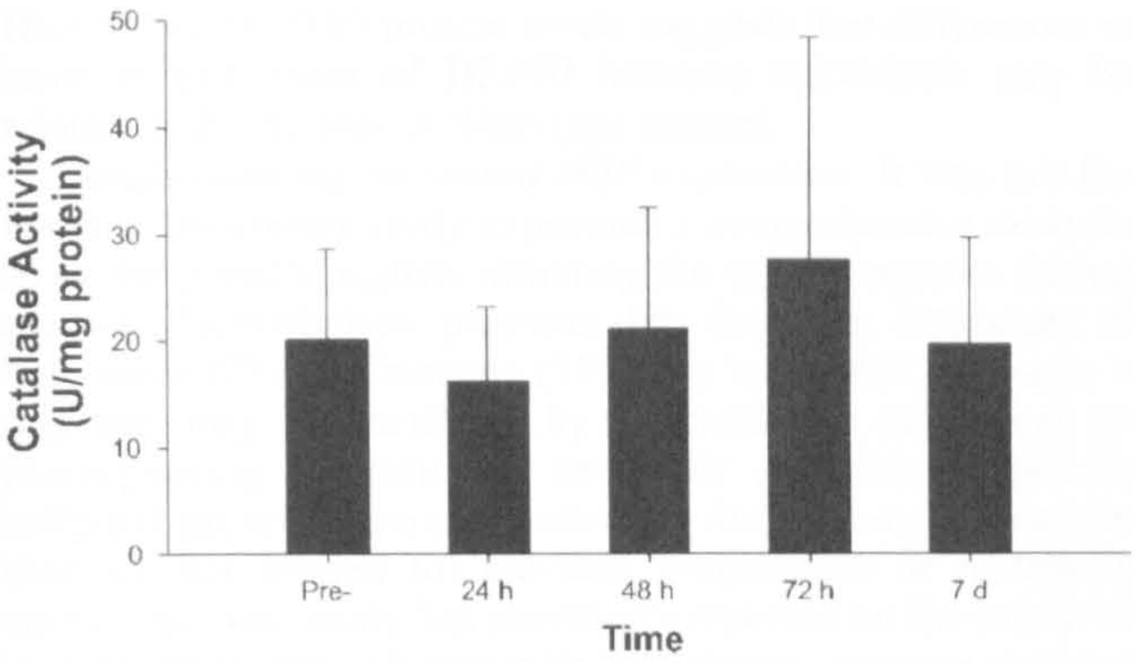

Fig. 7. Catalase activity of the vastus lateralis muscle before and after exercise.

HSP genes occurs during (9), immediately after (36), or several hours postexercise (47), HSP translation appears to require greater than 24 h. It is, therefore, possible that a stress response may have also been observed in these studies, had multiple postexercise biopsies been performed.

The increase in muscle HSP70 and HSP60 content in the present study is somewhat smaller than that previously observed following one-legged cycling (17, 18). Of note, however, is the difference in training status of the subject groups between studies. Although the present subjects were not specifically trained athletes, they were involved in an average of 2 h of physical activity per week (e.g., recreational sport), significantly more activity than the sedentary lifestyles led by the subjects involved in the previous investigations. It may therefore be speculated that sedentary subjects may mount a greater stress response than that of active or trained subjects (whose muscles are somewhat more preconditioned to exercise stresses) so as to combat any homeostatic disruption evoked by the exercise stress. This is true in the muscles of rodents, whereby the exercise-induced increase in HSP70 in rat soleus muscle is higher in untrained rats than endurance-trained rats (41). In a similar manner to the HSP70 response, the lower HSP60 response observed here may also be due to the differing characteristics of active and sedentary subjects. For example, the mitochondria of active subjects are likely to be more equipped with other endogenous defense mechanisms to cope with the changes in oxygen flux that occur during exercise. In such cases, a dramatic HSP60 response may therefore not need to be mounted. In line with this is the increased baseline activity of total SOD activity in the present subjects compared with the sedentary subjects studied by Khassaf et al. (17, 18).

Although our data displayed individual variation in both the time course and magnitude of HSP70 responses (see individual Western blots, Fig. 2, *C–E*), this variation was substantially lower than that previously demonstrated by Khassaf et al. (17). We believe this to be attributable to the more "tightly controlled" exercise protocol utilized in the present study. When exercising at the lactate threshold (as opposed to percentage of $\dot{V}O_{2\,max}$), relatively homogenous physiological (both cardiac and metabolic) responses are observed (2). This is true even when comparisons are made between trained and untrained subjects (2). These authors speculated that such homogenous responses may be due to similar fiber-type recruitment patterns. The latter appears relevant in an evaluation of the stress response considering data from rodent studies suggesting that HSP70 expression in the skeletal muscle of rodents following treadmill running displays an intensity-dependent relationship that is partly reflective of muscle recruitment patterns (31).

Small-HSP response. The small HSPs are thought to play an important role in the remodeling of myofibrillar structure following stressful and damaging insults (34). It has therefore been suggested that the small HSPs may be particularly active in the recovery process following exercise-induced muscle damage. In agreement with this, an increased expression of HSP27 has been consistently observed at 48 h after lengthening contractions of the biceps (42–44). Feasson et al. (8) also observed an approximate twofold increase in both HSP27 and αB-crystallin at 24 h following a 30-min downhill running protocol. In contrast to the above studies, the present data revealed no increases in muscle content of the small HSPs. This may be due, in part, to the nondamaging nature of our exercise protocol that does not appear to cause any overt structural or functional damage in active young male populations. It is possible, therefore, that whereas the HSP70 and HSP60 proteins may be upregulated during exercise by oxidative, thermal, metabolic or cytokine signals, the small HSPs are more responsive to contractile-induced mechanical stresses. It may also be that the relatively high baseline levels of both small HSPs were already sufficient to counteract any stresses that the contractile and structural proteins encountered during exercise. At present, data concerning the exercise-induced expression of small HSPs in human skeletal muscle are extremely limited, and thus further descriptive studies characterizing a response to various exercise protocols appear warranted.

Baseline stress protein levels. Of all baseline protein levels examined, only HSP70 and MnSOD showed large individual variation. This interindividual variation in resting muscle HSP70 levels is not novel and has been demonstrated previously (17). Data from rodent studies have shown that HSP70 is preferentially expressed in muscles with a high proportion of oxidative fibers (13, 16, 22, 23). It is possible, therefore, that the variations in baseline HSP70 levels observed here may be due to differing fiber-type characteristics between subjects. We were unable to quantify muscle fiber types due to the small tissue samples obtained by our chosen biopsy technique. Nevertheless, the observation of moderate correlations between

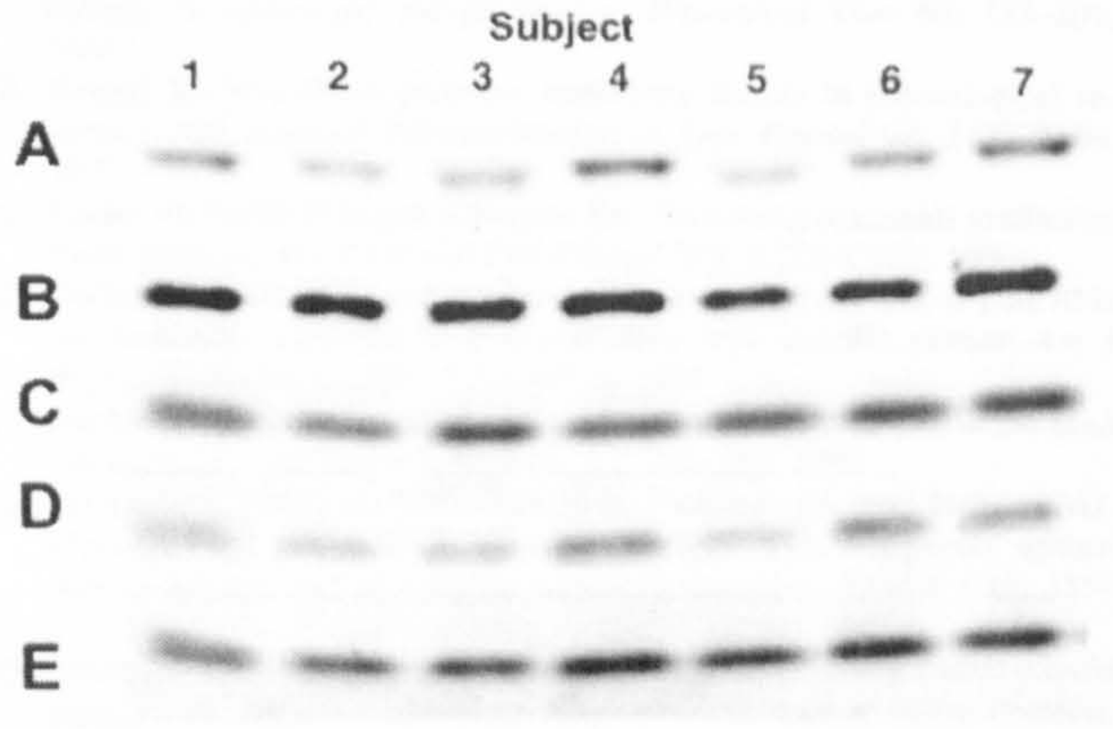

Fig. 8. Western blots showing resting muscle content of HSP70 (*A*), HSC70 (*B*), HSP27 (*C*), MnSOD (*D*), and αB-crystallin (*E*).

HSP70 and MnSOD protein levels suggests that differences in baseline expression of HSP70 between individuals may be related to differences in fiber-type content.

Signals inducing increased HSP expression. It was not the intent of the present study to provide a comprehensive analysis as to the possible signals initiating the stress response during exercise. Nevertheless, previous data from our laboratory in both mice (27) and humans (17) have suggested that such a response may be mediated by an oxidative stress and an accompanying transient and reversible oxidation of protein sulfydryl groups. Alternate activators during exercise include (but are not limited to) elevated temperature or metabolic stress. Only one study has previously reported on the extent of muscle temperature change with their chosen exercise protocol (9). In the present study, we observed a large increase in both muscle (4°C) and core (2°C) temperature, demonstrating a local and systemic hyperthermia effect. At present, we therefore feel it difficult to conclusively dismiss the role of increased muscle and core temperature in contributing to the exercise-induced production of HSPs in human skeletal muscle.

Biological significance of increased HSP expression. An increased muscle content of HSPs following exercise is thought to restore cellular homeostasis, promote cellular remodeling, and provide cytoprotection against further insults (25). Data from our laboratory in rodent models using preconditioning stresses (26, 28) or transgenic approaches (24) have demonstrated that HSPs provide increased protection against contraction-induced damage. A diminished HSP response to contractile activity has also been observed in muscles of old rats (46) suggesting that stress proteins may, in part, play an important role in maintaining muscle function during the ageing process. Indeed, muscles of old transgenic mice overexpressing HSP70 displayed an enhanced recovery of muscle function following a period of damaging contractions compared with muscles of old wild-type mice (24). Further studies are required to examine whether such a biological role of increased muscle HSP levels exists in a human model.

In summary, the present data demonstrate that the skeletal muscle of healthy active young male subjects responds to a period of moderately demanding and nondamaging running exercise via an upregulation of several HSPs (predominantly HSP70). Given the well-documented cytoprotective role of these proteins, it is possible that their increased expression following exercise functions to restore cellular homeostasis and to offer increased protection against further stressful insults. Further studies are required to examine the differing HSP responses to varying types of exercise protocols in specific subject populations (i.e., age, gender, and training status specific), to investigate possible mechanisms of activation, and to determine the precise biological significance of this increased expression.

ACKNOWLEDGMENTS

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Appendix III

Conference Communication I

Morton, J.P., Atkinson, G., MacLaren, D.P.M., Cable, N.T., Gilbert, G., McArdle, A., Broome, C. and Drust, B.

Reliability of maximal muscle force and voluntary activation as markers of exercise-induced muscle damage

The Physiological Society, King's College London Meeting, December 2004.

J Physiol, **565P**, PC10.

m, P⟨0.05, paired t test), with a similar improvement in WD. Following a period of 2 weeks rest, improvements in CD and WD had waned by approximately 15 m. A repeat regimen of stimulation (Bout2) reversed the decline in CD and WD after the rest period, and reinstated values back to those seen after the first stimulation period. Lower limb blood flow increased from baseline after Bout1 (3.5±0.6 vs. 2.1±0.3ml.min^{-1} (100ml)$^{-1}$, P⟨0.05) and did not decline over 2 weeks rest. Flow increased further after Bout2 (5.7±1.0ml.min^{-1} (100ml)$^{-1}$), and remained 92% above pre-treatment values at the end of the study. TENS did not induce improvements on any measure.
Chronic low frequency electrical stimulation of ischaemic calf muscles is therefore an effective stimulus for transient improvements in functional capacity in PVD. Applied intermittently, it enables improved functional capacity to be sustainable. We also show that stimulation provides amelioration of the haemodynamic compromise experienced by claudication patients

Tsang GMK et al. (1994). Eur J Vasc Surg 8, 419-422.

Anderson SI et al. (2004). Eur J Vasc Endovasc Surg 27, 201-209.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC10

Reliability of maximal muscle force and voluntary activation as a marker of exercise-induced muscle damage

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The loss of the ability of skeletal muscle to generate force is one of the most appropriate and valid means by which to quantify muscle damage. However, measurements of maximal muscle force may include many potential sources of error, the most common of which is the lack of central drive to the muscles. The aim of the present study was to therefore determine the reliability of maximal isometric quadriceps muscle force and voluntary activation over a time-scale that is typically employed to examine the aetiology of exercise-induced muscle damage. We also characterised the reliability of several twitch interpolation variables and determined the effects of different calculation methods on estimates of voluntary activation, specifically the central activation ratio (CAR) and interpolated twitch ratio as calculated from both unpotentiated (ITTUNPOT) and potentiated twitches (ITTPOT).
Eight healthy active males, mean (SD): age 21.4 (0.9) years; mass 83.6 (8.2) kg; height 179.8 (2.4) cm performed repeated maximal voluntary isometric contractions (MVC) of the quadriceps over a 7 day period (baseline and 2h, 6h, 24h, 48h, 72h, 7 days post). Differences across the 7 sessions were assessed using repeated measurements ANOVA. Ninety-five percent repeatability coefficients were also calculated according to Bland and Altman (1999). There was a significant difference (P<0.05) in estimates of voluntary activation between calculation methods with values of 98.9, 91.2 and 94.6% calculated for the CAR, ITTUNPOT and ITTPOT methods, respectively. The ITTPOT was considered the most valid estimate of voluntary activation. There was no evidence of any systematic changes over time in maximal muscle force, voluntary activation (ITTPOT), interpolated twitch, unpotentiated twitch or potentiated twitch (P>0.05) where 95% repeatability coefficients of 76.03 N, 4.42%, 8.44 N, 25.92 N and 43.58 N were observed, respectively.
These data indicate that young healthy well-familiarised subjects can reproduce their perceived maximal efforts both within and between days where activation levels of >90% are routinely achieved. Providing activation remains within these limits in the 7-days following an acute bout of exercise, the researcher may be 95% certain that exercise-induced muscle damage is present in individual subjects (taken from similar subject populations) if MVC force falls outside these limits.

Bland J.M. & Altman D.G. (1999). Stat Methods Med Res 8, 135-160.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC11

Responses to the perturbation of the leg during walking on a treadmill in elderly women fallers and non-fallers

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We have previously described two recovery strategies after perturbations of gait during treadmill walking. Fit older subjects more frequently used the strategy that appeared less stable than did the young (Bruce et al, 2000). We have now compared recovery strategies in elderly subjects who had recently fallen with those who had not.
Subjects were 24 women, mean age 74.2 (sd 3.4) years. All had fallen at least once in the last four years and had participated in exercise tailored to improve balance and gait. The "fallers" group was those 16 subjects who had fallen one or more times in the previous year. The study had RNOHT ethical committee approval; subjects gave written informed consent following Helsinki guidelines.
Subjects wore a safety harness and walked on a PowerJog GX100 treadmill at their own comfortable walking pace, which was the same for each group. Kinematics were recorded by the CODA mpx30 motion analysis system using 16 LED markers. At the beginning of swing phase during randomly selected strides CODA triggered the perturbations, applied by cords attached to each foot. The perturbations were not large enough to cause loss of balance.
We obtained 44 gait perturbations (each the average of 6 trials) from the fallers and 19 from the non-fallers. Each subject adopted a consistent strategy in response to perturbation (Bruce et al, 2000). The timing of lifting of the un-tripped leg immediately after the perturbation was delayed in a gretaer proportion of the fallers (14/44 fallers vs 1/19 non-fallers, Chi squared, p=0.023). Fallers took longer to return to their normal pattern

Appendix IV

Conference Communication II

Morton, J.P., MacLaren, D.P.M., Cable, N.T., Baker, A., McArdle, A. and Drust, B.

The development of a 'non-damaging' treadmill exercise protocol as a stimulus to initiate the exercise-induced expression of heat shock proteins in human skeletal muscle

The University of Liverpool Mini-Symposium on ROS, Muscle and Ageing, University of Liverpool, May 2005.

The development of a non-damaging treadmill exercise protocol as a stimulus to initiate the exercise-induced expression of heat shock proteins in human skeletal muscle

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Interpretation of heat shock protein data from human studies is complicated by the disparate exercise protocols utilised by investigators. This is particularly the case in those instances where there is a damaging component to the exercise protocol where the inflammatory response resulting from damage may further increase the intra-muscular expression of heat shock proteins (HSPs). The use of 'non-damaging' exercise protocols to initiate the exercise-induced expression of HSPs provides a more controlled methodological approach whereby any increased expression of HSPs is more likely to have arisen from factors occurring solely during exercise. The aim of this study was to develop a 'non-damaging' treadmill exercise protocol to utilise as a stimulus to initiate the exercise-induced expression of HSPs in human skeletal muscle.

Six 'active' (age, 25 ± 2 years; mass, 76.3 ± 7.6 kg; height, 1.78 ± 0.04 m; $\dot{V}O_{2max}$, 56.4 ± 3.3 $ml.kg^{-1}.min^{-1}$; lactate threshold, 69.3 ± 2.1 % $\dot{V}O_{2max}$) and six 'trained' (age, 25 ± 5 years; weight, 74.3 ± 7.9 kg; height, 1.74 ± 0.06 m; $\dot{V}O_{2max}$, 66.4 ± 5.4 $ml.kg^{-1}.min^{-1}$; lactate threshold, 76.8 ± 1.4 % $\dot{V}O_{2max}$) males performed 45 min of treadmill running at speeds corresponding to their lactate threshold (11.2 ± 0.7 and 13 ± 0.9 km for active and trained, respectively). The exercise protocol resulted in no significant reductions ($P>0.05$) in the force generating capability of the quadriceps muscle as determined by maximal isometric force. The exercise protocol did result in statistically significant ($P<0.01$) increases in serum creatine kinase (CK) activity (75.8 ± 22.9 and 96.3 ± 16.4 $IU.L^{-1}$ for active and trained, respectively) and ratings of perceived quadriceps soreness, as determined by a visual analogue scale (12.6 ± 11.6 and 11.8 ± 13.5 mm for active and trained, respectively). These responses peaked at 24 h post-exercise in both groups. These small increases in CK and ratings of perceived quadriceps soreness are, however, clinically insignificant in terms of those values typically associated with damaging interventions (Close *et al.*, 2004). Two subjects (1 trained and 1 active) re-performed the exercise protocol 6-8 weeks later where muscle biopsies were obtained from the vastus lateralis immediately pre- and 48 h post-exercise. Post-exercise HSP60 and HSP70 muscle content increased to 122 – 142 and 124 – 145% of pre-exercise values, respectively.

It is concluded that 45 min of treadmill running at an intensity corresponding to the lactate threshold is essentially 'non-damaging' in nature. This exercise protocol is also sufficient to induce HSP expression in the skeletal muscle of both active and trained young male subjects. This protocol will therefore be utilised in future research as a 'non-damaging' exercise protocol to further study the exercise-induced regulation of HSPs in human skeletal muscle.

Close, G.L., Ashton, T., Cable, T., Doran, D. and MacLaren, D.P.M. (2004). *Eur J Appl Physiol*, **91**, 615-621.

Appendix V

Conference Communication III

Morton, J.P., MacLaren, D.P.M., Cable, N.T., McArdle, A. and Drust, B.

Time-course and differential expression of heat shock proteins in human skeletal muscle following non-damaging treadmill exercise: is heat a mechanism of activation?

11th Annual Congress of the European College of Sport Science, Lausanne, Switzerland, July 2006.

cantly affected by the COX-2 inhibitor. However, similar findings are reported by Peterson et al. (2003) after administrating other types of anti-inflammatory drugs.

Connolly et al. (2003). J.Strength.Cond.Res. 17, 197-208
Peterson et al. (2003). Med.Sci.Sports Exerc. 35, 892-896.

TIME-COURSE AND DIFFERENTIAL EXPRESSION OF HEAT SHOCK PROTEINS IN HUMAN SKELETAL MUSCLE FOLLOWING NON-DAMAGING TREADMILL EXERCISE: IS HEAT A MECHANISM OF ACTIVATION?

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The exercise-induced expression of heat shock proteins (HSPs) as a cellular stress response in rodent models is relatively well defined (McArdle et al., 2001). Comparable data from human studies, however, are limited and the mechanisms contributing to the exercise-induced production of HSPs are far from understood. Although exercise-associated hyperthermia is routinely cited as a possible signal responsible for inducing an increased production of HSPs following exercise, this hypothesis has not been formally tested in human skeletal muscle. The aim of the present study was to therefore characterise the time-course of response of the major HSP families in human skeletal muscle following a running exercise protocol and to test the hypothesis that increased muscle and core temperature mediate the increased production of HSPs following exercise.

Eight healthy active males performed 45 min of running exercise at a treadmill speed corresponding to their lactate threshold. Muscle biopsies were obtained from the vastus lateralis muscle immediately prior to and at 24 h, 48 h, 72 h and 7 days post-exercise. Seven healthy active males also underwent a passive heating protocol of 1 h duration in which one leg was immersed in a tank containing hot water (45 °C). The contra-lateral limb remained outside the tank and was not exposed to heat stress thus serving as a control leg. Muscle biopsies were obtained from the vastus lateralis of both legs immediately prior to and at the time of peak HSP expression induced by exercise (observed to be at 48 h and 7 days post-heating). The exercise and heating protocol induced significant increases ($P<0.05$) in rectal (1.7±0.3 and 1.5±0.2 °C, respectively) and muscle temperature (3.8±0.8 and 3.7±0.6 °C, respectively). Muscle temperature of the control leg showed no significant change following heating (pre: 36.1±0.5, post: 35.6±0.4 °C). Exercise induced a significant ($P<0.05$) but variable increase in HSP70, HSC70 and HSP60 expression with peak increases (typically occurring at 48 h post-exercise) of 210±70%, 170±75% and 139±23% of pre-exercise levels, respectively. In contrast, heating failed to induce a significant increase in muscle content of these proteins. Neither heating nor exercise induced a significant increase in HSP27, αB-crystallin, MnSOD protein content or the activity of superoxide dismutase and catalase.

This study is the first in which the time-course and extent of HSP production in human skeletal muscle have been characterised following a moderately demanding running exercise protocol. Data demonstrate that the skeletal muscle of healthy active young males adapts to such stresses via an increased production of several HSPs (predominantly HSP70) where peak responses typically occur at 48 h post-exercise. We conclude that increases in both systemic and local temperature per se appear not to be mediating this response.

McArdle, A. et al. (2001). Am J Cell Physiol, 280: C621-C627

EFFECT OF A FED OR FASTED STATE ON THE SALIVARY IMMUNOGLOBULIN A RESPONSE TO EXERCISE

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It has been previously suggested that the nutritional status of an individual can influence the levels of salivary immunoglobulin A (s-IgA) at rest, yielding higher and more variable concentrations in fasting saliva compared to non-fasting saliva (Gleeson et al., 2004). Prolonged, strenuous exercise has been associated with changes in the levels of this antibody (Gleeson et al., 2003) however, the influence of the fed or fasted state on the s-IgA response is unknown. Thus, the present study investigated the effect of a fed or fasted state on the s-IgA response to prolonged cycling. Using a randomised cross-over design, 8 males and 8 females of mixed physical fitness, mean (SEM) age 22 (1) years, performed 2 hours cycling on a stationary ergometer at 65% of their maximal oxygen uptake on one occasion following an overnight fast (FAST) and on another occasion following the consumption of a 2.2 MJ high carbohydrate meal (FED) 2 hours before. Timed, unstimulated whole saliva samples were collected immediately before ingestion of the meal, immediately pre-exercise, at 5 minutes before cessation of exercise, immediately post-exercise and at 1 hour post-exercise. The samples were analysed for s-IgA concentration, osmolality and cortisol and saliva flow rates were determined to calculate the s-IgA secretion rate. Salivary data were analysed using a two factor repeated measures ANOVA and post hoc t-tests with Holm-Bonferroni adjustments were applied where appropriate. Carbohydrate oxidation was significantly higher ($P<0.05$) and fat oxidation tended to be lower ($P=0.06$) in FED compared to FAST. Saliva flow rate decreased during exercise from 0.50 (0.04) mL/min to 0.37 (0.03) mL/min ($P<0.05$), s-IgA concentration increased during exercise from 163 (20) to 232 (24) mg/L ($P<0.05$) but the s-IgA secretion rate remained unchanged, pre-exercise: 79 (11) µg/min, post-exercise· 74 (9) µg/min. There was a significant reduction in the s-IgA:osmolality ratio from 2.3 (0.2) pre-exercise to 1.5 (0.1) post-exercise ($P<0.05$). Salivary cortisol increased from 9.9 nmol/L (1.6) pre-exercise to 16.7 (2.9) nmol/L post-exercise ($P<0.05$). There was no effect of FED versus FAST on these salivary responses. The immediately pre-exercise values of s-IgA were 165 (27) and 161 (27) mg/L in the FED and FAST trials, respectively. The s-IgA concentration and secretion rate were found to be significantly lower in females than in males across all time points ($P<0.05$); however, there was no significant difference between genders in the saliva flow rate or the s-IgA:osmolality ratio. These data demonstrate that the nutritional status does not influence resting s-IgA or the s-IgA response to prolonged exercise. Furthermore, these data suggest a significant effect of gender on resting s-IgA levels without affecting the acute response to exercise.

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HEART RATE VARIABILITY THRESHOLDS AND HEART RATE DEFLEXION POINT DURING MAXIMAL RUNNING EXERCISE IN CIRCUMPUBERTAL BOYS: ACCURATE METHODS TO ASSESS VENTILATORY THRESHOLDS?

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Together with maximum oxygen uptake, ventilatory thresholds (VT1 and VT2) are the physiologic measures the most used to characterise aerobic fitness. To approach VTs without gas analysis, some authors have proposed to use the heart rate profile during exercise, while