Skeletal muscle as an endocrine organ: impact of muscle-derived cytokines and extracellular heat shock proteins

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Abstract

Skeletal muscle wasting in the critically ill with sepsis is intrinsically linked to the elevations in circulating levels of cytokines characteristic to systemic inflammation and this effect may be exacerbated in older people. A key cytokine in this hyper-inflammatory state is TNF-α. Muscle is a plastic organ which adapts to stresses, including systemic inflammation in a number of ways, including the increased expression of Heat Shock Proteins (HSPs) and increased expression and release of cytokines (myokines). The release of HSPs by non-muscle cells has been described, but the extracellular function of HSPs is poorly understood. The aims of this thesis were to determine the effect of TNF-α on HSP and cytokine release by muscle; to elucidate the mechanism of HSP release from skeletal muscle, to identify the autocrine and paracrine signalling properties of eHSPs and to determine the impact of ageing on the ability of skeletal muscle to act as an endocrine organ.

C2C12 myotubes were treated with TNF-α and cells and media were examined for HSP and cytokine content. The mechanisms of HSP and cytokine release were examined which included exosomal and golgi-mediated processes. The paracrine effects of muscle-derived HSP60 were determined by treatment of myotubes or SaOs-2 osteoblast cells with HSP60 at concentrations comparable to serum levels reported in critically ill patients. To determine the effect of TNF-α on the release of HSP60 and cytokines from muscles of old mice, isolated muscle fibres from the FDB of adult and old mice were treated with TNF-α.

Treatment of C2C12 myotubes with TNF-α resulted in increased content of HSP10, HSP60 and HSP70 and several cytokines. TNF-α treatment resulted in the specific release of HSP60 as well as IL-6, MCP-1, KC and RANTES from myotubes into the cell culture media. Inhibitor studies demonstrated that the release of HSP60 occurred via exosomes whereas cytokines were released by golgi-mediated transport. Treatment of myotubes with HSP60 resulted in the release of IL-6, MCP-1 and RANTES by muscle cells but had no effect on markers of bone formation in SaOs-2 osteoblast cells. Treatment of C2C12 myotubes or SaOs-2 osteoblast cells with muscle-derived exosomes had no effect on cytokine release or markers of bone formation respectively. Comparison of the amount of HSP60 released by muscle in exosomes to levels reported in pathological states suggest that muscle is not a major source of HSP60. In contrast, comparison of the levels of cytokines released by muscle suggest that muscle is likely to be a major source of these cytokines in the critically ill. Treatment of isolated fibres from the FDB muscle of adult WT mice with TNF-α resulted in the significant increase in HSP60, IL-6 and KC in the media. In contrast, no effect was seen on the media content of HSP60 following treatment of isolated fibres from old WT mice although the media content of cytokines was comparable to that from fibres of adult WT mice.

Data suggest that muscle can act as a significant source of cytokines in response to elevated levels of TNF-α and this may have significant implications for treatment of the critically ill. Muscle can release HSP60 as part of an exosomal process although the levels of HSP60 released are lower than those required to activate cytokine production and release by muscle or osteoblast cells. In contrast, muscle can act as a significant contributing source of cytokines in response to elevated levels of HSP60 derived from other cell types.

CONTENTS

CONTENTS

CHAPTER 3: THE EFFECT OF TNF-α TREATMENT ON THE HEAT SHOCK RESPONSE IN C2C12 MYOTUBES; MUSCLE AS A SOURCE OF EXTRACELLULAR HSPS?

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

LIST OF ABBREVIATIONS

17AAG – 17-(allylamino)-17-demethoxygeldanamycin
ADP – Adenosine Diphosphate
ATP – Adenosine Triphosphate
AP – Alkaline Phosphatase
APS – Ammonium persulphate
BCA – Bicinchonic acid
BFA – Brefeldin-A
BSA – Bovine serum albumin
CCL – Chemokine ligand
cDNA – Complementary deoxyribose nucleic acid
CD9 – Cluster of differentiation 9
CD63 – Cluster of differentiation 63
CD81 – Cluster of differentiation 81
CCL2 – Chemokine ligand 2 (MCP-1)
CCL5 – Chemokine ligand 5 (RANTES)
CXCL1 – Chemokine (CXC motif) ligand 1 (KC)
CCR – Chemokine receptor
DMA – Dimethyl amiloride
DNA – Deoxyribose nucleic acid
DPBS – Dulbecco's phosphate buffered saline
eHSP – extracellular heat shock protein
ELISA – Enzyme-linked immunosorbant assay
FGF-basic – Fibroblast growth factor-basic (FGF-2)
GM-CSF – Granulocyte-macrophage colony stimulating factor
HSP10 – Heat shock protein 10
HSP25 – Heat shock protein 25
HSP60 – Heat shock protein 60
HSC70 – Heat shock cognate 70
HSP70 – Heat shock Protein 70
HSP90 – Heat shock protein 90
IFN-γ – Interferon-gamma
IL-1β – Interleukin-1 Beta
IL-1ra – Interleukin-1 receptor antagonist
IL-2 – Interleukin-2
IL-4–Interleukin-4
IL-5 – Interleukin-5
IL-6 – Interleukin-6
IL-8 – Interleukin-8
IL-10 – Interleukin-10
IL-12 – Interleukin-12
IL-13- Interleukin-13
IL-15 – Interleukin-15
IL-17 – Interleukin-17
KC – Keratinocyte chemoattractant (CXCL1)
LAL – Limulus amoebocyte lysate
LPS – Lipopolysaccharide

MOF- Multiple Organ Failure
MIG – Monokine induced by gamma interferon
MIP-1α – Macrophage inflammatory protein 1-alpha
MCP-1– Monocyte chemotactic protein-1 (CCL2)
MHC-II – Major histocompatibility complex class II
MVB – Multi-vesicular body
NFκB – Nuclear Factor kappa B
OC - Osteocalcin
PBS – Phosphate buffered saline
qPCR – Quantitative polymerase chain reaction
P1NP - Procollagen 1 n terminal peptide
RANTES – Regulated upon Activation, Normal T-cell Expressed, and Secreted (CCL5)
RNA – Ribose nucleic acid
SDS – Sodium dodecyl sulphate
SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis
si-RNA – Short-interfering RNA
sTNF-α –Soluble TNF-α receptor
TEMED – NNN'N'-tetramethylethylene-diamine
TRADD – TNF-receptor associated death domain
TRAF –TNF receptor-associated factor
TSG-101 – Tumour susceptibility gene 101
TLR-2 – Toll-like receptor 2
TLR-4 – Toll-like receptor 4
TNF-α – Tumour necrosis factor alpha
VCAM – Vascular cellular adhesion molecule
WT – Wild type

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CHAPTER 1

INTRODUCTION

Skeletal muscle suffers profoundly during sepsis, with a substantial loss of muscle mass and function which impacts significantly on clinical outcome. Inflammation is described as the main driver of skeletal muscle atrophy in the critically. Skeletal muscle is proposed to be a source of cytokines and it is hypothesised that they play a role in the hyperinflammatory state presented in the critically ill. The overall aim of the work carried out in this thesis was to investigate the role of skeletal muscle as an endocrine organ during systemic inflammation; with a perspective towards the critically ill septic individual. This chapter details the background behind inflammation in the critically ill, the role skeletal muscle has to play in this inflammation and the potential mechanisms involved.

1.1 Skeletal muscle structure and function.

Human physiology has evolved over thousands of years and this success has been underpinned by the function of skeletal muscle. As a robust and plastic organ, muscle not only provides force for locomotion but also produces heat to maintain body temperature. Skeletal muscle is the largest protein store in the body comprising 50% total protein and 40% total body weight (MacIntosh, 2006). The architecture of skeletal muscle is characterised by the bundles of muscle fibres organised along the length of the muscle. Each fibre bundle is comprised of numerous filaments termed myofibrils. Each myofibril is comprised, in a sequential fashion, of the contractile proteins actin and myosin.

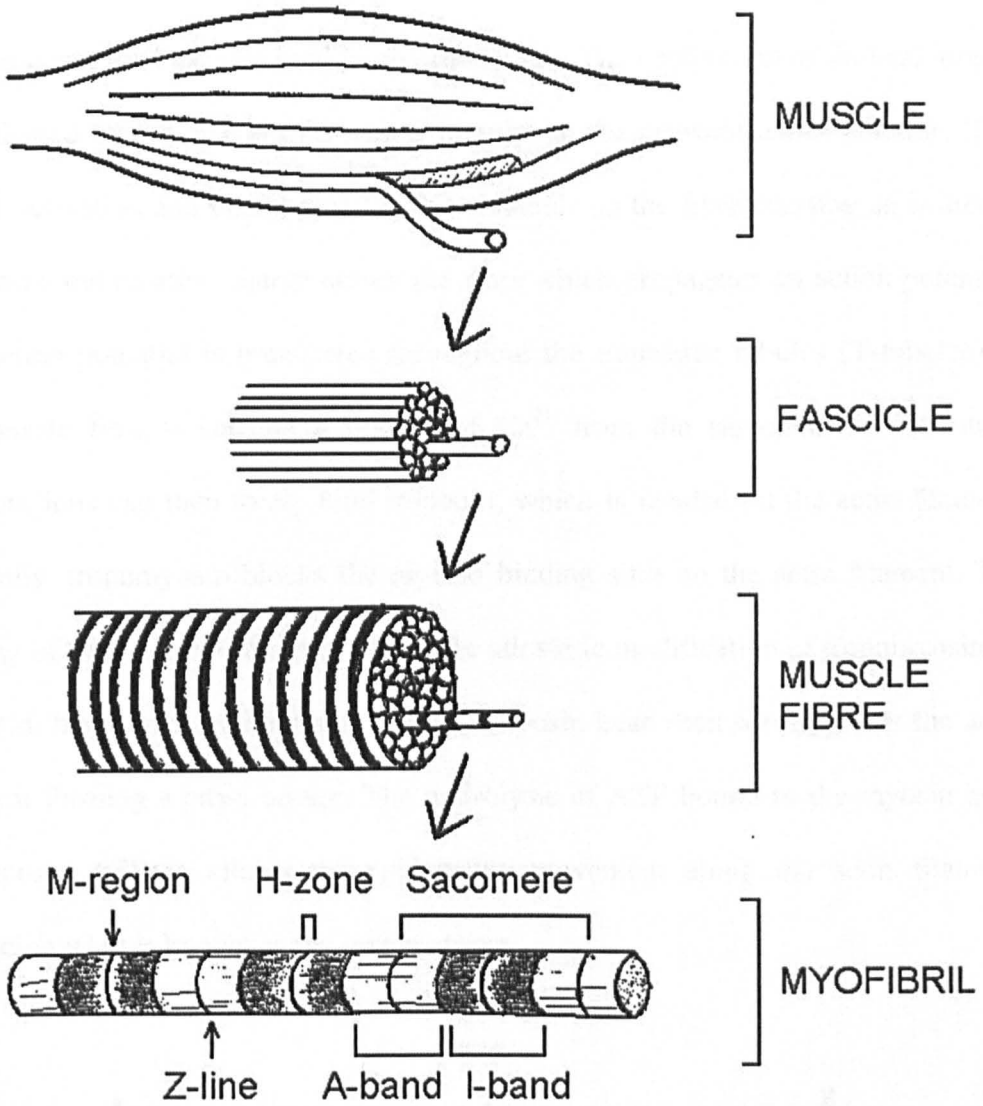


Figure 1.1 representation of the hierarchical organisation of skeletal muscle. Image adapted from (MacIntosh, 2006).

The organisation of the actin and myosin filaments is termed the sarcomere, and this gives skeletal muscle its characteristic striated appearance (Figure 1.1). The actin and myosin filaments that comprise the sarcomere allow skeletal muscle to contract and generate force (Huxley and Hanson, 1954).

The processes involved in the contraction of skeletal muscle are highly specialised and stringent, to allow maximal force production. The contraction of skeletal muscle is instigated by the arrival of a neural impulse to the neuromuscular junction. This causes activation and opening of Na^+/K^+ channels on the fibre, causing an influx of Na^+ and a net positive charge across the fibre which propagates an action potential. The action potential is transferred throughout the transferse tubules (T-tubules) of the muscle fibre – causing a release of Ca^{2+} from the sarcoplasmic reticulum. Calcium ions can then freely bind troponin, which is located on the actin filament. Typically, tropomyosin blocks the myosin binding sites on the actin filament. The binding of Ca^{2+} to troponin then allows the allosteric modification of tropomyosin by troponin, freeing up the binding sites. The myosin head then readily binds the actin filament forming a cross-bridge. The hydrolysis of ATP bound to the myosin head by myosin ATPase allows the ratchet-like movement along the actin filament, producing what is known as the power stroke.

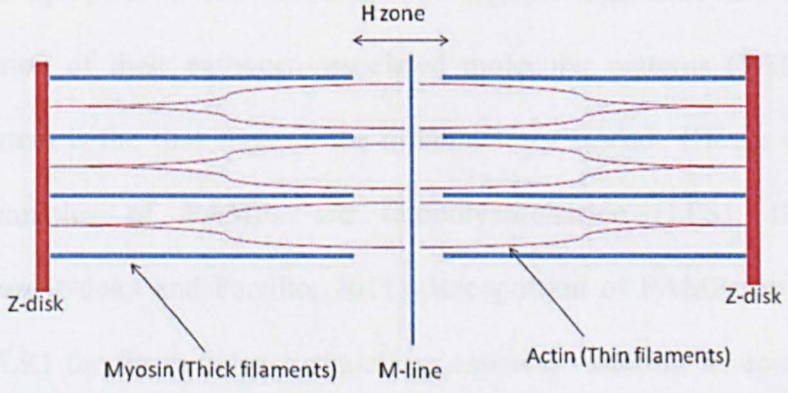


Figure 1.2 Schematic representation of the sarcomere.

This is a cyclical, ATP-dependent process, whereby myosin can re-bind ATP and move to the next binding site (approx. 10nm) again under the control of ATP hydrolysis. The length of the actin and myosin filaments remains constant

throughout the contraction, and instead they slide past each other, indicated by the sarcomere shortening at the z-disks (Figure 1.2).

Pivotally important during "hunter-gatherer" times, skeletal muscle still has an equally significant role to play in a more sedentary modern age. Muscle is a plastic organ which can hypertrophy in response to exercise and atrophy in the critically ill. Inflammation in the critically ill is suggested to be one of the main drivers of the loss of skeletal muscle mass and function.

1.2 The Pathophysiology of sepsis

Sepsis is a leading cause of morbidity in the intensive care setting. Sepsis is clinically defined as the presence of a hyperinflammatory state accompanied by tachycardia and increased respiration (Lanone et al., 2005). The primary cause of sepsis is usually a severe gram-negative bacterial infection, however recent observations have shown there is a rising prevalence of gram-positive infections (van der Poll and Opal, 2008). The invasion of pathogenic organisms into the body and the recognition of their pathogen-associated molecular patterns (PAMPs) by the immune system is the first stage in the inflammatory cascade (Bozza et al., 2007). Typical examples of PAMPs are lipopolysaccharide (LPS), flagellin and peptidoglycan (Nduka and Parrillo, 2011). Recognition of PAMPs by the toll-like receptor (TLR) family initiates a signalling cascade, resulting in up-regulation of transcription factors such as nuclear factor kappa B (NFκB) and release of pro-inflammatory cytokines (Bozza et al., 2007). This array of cytokines typically recruits immune cells to the site of infection to combat the bacterium. However, in sepsis the overwhelming bacterial infection causes an uncontrolled hyperinflammatory state which is poorly understood (Ebong et al., 1999). This

hyperinflammatory state exposes cells and tissues throughout the body to a large repertoire of pro-inflammatory cells, reactive oxygen species (ROS) and cytokines (Gabay and Kushner, 1999). Typically, elevated circulating levels of pro-inflammatory cytokines such as TNF-α and IL-1β are correlated with poor clinical outcome in the critically ill (Casey et al., 1993). Moreover, over time the culmination of the chronic inflammatory is multiple organ failure (MOF).

1.2.1 The role of skeletal muscle in sepsis

A key organ affected by the hyper-inflammatory state during sepsis is skeletal muscle. Typically, patients with sepsis in the intensive care unit (ICU) suffer profound loss of muscle mass and function (Lightfoot et al., 2009). Loss of skeletal muscle in sepsis patients is a clear determinant of morbidity in this population (Griffiths, 1996). In sedated and ventilated patients lean body mass declines at a rate of 0.5-1.0% per day compared with non-septic sedentary control patients (Ferrando et al., 1996). Patients with severe sepsis lose up to 16% of total body protein over a 3-week period, contributing to an overall loss of approximately 3kg of skeletal muscle mass (Finn et al., 1996). Length of stay in ICU is correlated with significant reduction in muscle fibre cross-sectional area (CSA) (Helliwell et al., 1998).

1.2.2 Impact of ageing on muscle wasting in the critically ill.

The loss of muscle mass in the critically ill is often compounded by the fact that the median age for ICU admission is 65 years old, therefore as a result of ageing; a significant reduction in skeletal muscle mass is likely to have already occurred, compared with that of a young adult. This is believed to be one of the key determinants in prognosis for elderly patients in ICU (Griffiths, 1996). In addition the aberrant low-grade inflammation present in the elderly exacerbates the overall

hyperinflammatory state in the critically ill. Thus, contributing not only to the immune cascade but also the catabolism and weakness of skeletal muscle in critically ill individuals. The persistent inflammatory state in the elderly is also associated with muscle weakness. Studies examining the grip-strength of an elderly cohort found weakness correlated with markers of an inflammatory state (Beenakker et al., 2010).

1.2.3 Mechanisms of skeletal muscle wasting in the critically ill.

The loss of skeletal muscle in the critically ill is described as primarily due to an imbalance between rates of protein synthesis and degradation (Wolfe, 2005). Elevated pro-inflammatory cytokines and production of free radicals are typically associated with activation of proteolysis by disruption of the ubiquitin/proteasome pathway (Lang et al., 2007). Activation of atrogenes such as atrogin-1 and Forkhead box O 3 (FoxO3) activate protein breakdown by the proteasome and lysosome, and thus in turn induce atrophy of skeletal muscle (Zhao et al., 2008). Sepsis alters the contractile properties of muscle at the single fibre level, with a reduction in force generation also experienced at the whole muscle level (Supinski et al., 2000). The contractile proteins actin and myosin are most severely affected during periods of unloading, with atrophy inducing a significant reduction in the ratio of myosin to actin from 1.5 to 0.5, whereby there is a preferential loss of myosin (Norman et al., 2009). Studies have indicated a link between elevated levels of ROS, superoxide and nitric oxide in sepsis and loss of force generation in muscle (Callahan and Supinski, 2005). Loss of muscle mass and function contribute to weakness in these individuals. Weakness as a result of inactivity and chronic inflammation is associated with prolonged stays in ICU and poor clinical outcome (Chambers et al., 2009). Moreover, skeletal muscle weakness makes weaning patients off mechanical ventilation significantly more difficult. There is characteristic muscle wasting and

weakness of the diaphragm in the critically ill (Lanone et al., 2005). Treatment with glucocorticoids has also been described to up-regulate expression of atrogin-1 and MurRF-1 by down-regulation of PGC-1β, which may play an additional role in muscle wasting in the critically ill. Perturbations in mitochondrial function are also reported as key contributors to weakness and muscle dysfunction during sepsis (Fredriksson et al., 2006).

Overall, the loss of skeletal muscle mass and function in the critically ill has a severe impact clinically, but also contributes to the wider financial burden placed upon health services (Griffiths and Hall, 2010). The mechanisms by which sepsis results in loss of muscle mass and function are unclear but data suggest that they are compounded by the effect of physical inactivity on muscle loss in these patients.

1.2.4 Impact of physical inactivity on skeletal muscle dysfunction during sepsis.

Physical inactivity in the critically ill is also a significant factor in the progressive loss of muscle mass and function. Periods of prolonged bed rest have been described to have no clinical benefit or to improve patient outcome (Brower, 2009). Physical inactivity contributes to the mechanical unloading of muscle in the critically ill. The mechanical unloading of muscle is defined as low frequency and duration of muscle activation combined with exposure of muscle to smaller loads (Chambers et al., 2009). Mechanical unloading induces atrophy of skeletal muscle and a reduction in specific muscle force (Mazzatti et al., 2008). The unloading of muscle by either bed rest or mechanical ventilation and subsequent disuse muscle atrophy experienced in these individuals is associated with oxidative stress (Powers et al., 2007). The atrophy experienced during inactivity-induced unloading of muscle is associated with decreased protein synthesis (Thomason et al., 1989) in tandem with increased

protein degradation (Taillandier et al., 1996). Intervention using electrical stimulation of the quadriceps has shown no effect on preventing loss of muscle mass (Poulsen et al., 2011).

Physical inactivity has been described as a disease-like state, contributing to an array of co-morbidities such as cardiovascular disease and insulin resistance (Pedersen, 2009a). These conditions have significant implications on the survival of the critically ill in ICU. Studies into the beneficial effect of exercise have demonstrated that endurance training in rats caused up-regulation in skeletal muscle IL-10 expression in tandem with a down-regulation of TNF-α expression (Batista et al., 2010), suggesting that physical activity may promote an anti-inflammatory response, which may be physiologically beneficial.

1.2.5 Cytokine response in sepsis

An underlying bacterial infection in the critically ill is the driver for the chronic hyperinflammatory state present in these individuals. Fundamentally it is the array of cytokines downstream of the infection that causes the profound organ dysfunction. Studies of patients admitted to ICU show detectable levels of IL-1β, IL-6 and TNF-α in circulation. All three were shown to be predictors of mortality in these individuals (Casey et al., 1993). Examination of a wider array of cytokines in patients with severe sepsis, showed detectable levels of IL-1β, IL-3, IL-6,IL-7, IL-8, IL-10, IL-13, IFN-γ, MCP-1 and TNF-α. Significant correlation between elevated levels and mortality was associated with the cytokines, IL-4, IL-6 and IL-8 (Bozza et al., 2007). Rodent models of sepsis, using caecal ligation and puncture (CLP) have demonstrated elevated circulating levels of IL-1β, IL-6, KC and TNF-α which are correlated with severity of sepsis and mortality (Ebong et al., 1999). Studies have

suggested that elevated circulating cytokines can be measured as diagnostic markers in neonatal sepsis, with IL-1β, IL-6, IL-8 and TNF-α being the key predictors (Kurt et al., 2007). Furthermore, elevated circulating pro-inflammatory cytokines are correlated with the additional processes, indicative of sepsis, such as mitochondrial dysfunction, coagulation, apoptosis and muscle wasting and weakness (Cinel and Opal, 2009). However, attempts to blunt the cytokine response in sepsis in particular using antagonists have shown to be ineffective (Barratt-Due et al., 2010). In particular, the use of monoclonal antibodies to TNF-α in several clinical trials have shown to be largely ineffective (Abraham et al., 1998, Rice et al., 2006).Thus, although it is widely accepted that one of the key cytokines in mediating the systemic organ dysfunction and is TNF-α, treatment of such organ dysfunction remains elusive. This is likely to be due to the multi-functional nature of TNF-α.

1.3 Tumour necrosis factor-alpha.

TNF-α is encoded on the short arm of chromosome 6 in humans and 17 in mice (Spriggs et al., 1992). At 157 amino acids in length TNF-α exists in both soluble and membrane bound forms. TNF-α is initially produced as 26kDa membrane spanning protein, anchored in place due to a 79 amino acid precursor sequence. Subsequent proteolytic cleavage frees TNF-α from the membrane into a 17kDa soluble form (Vilcek and Lee, 1991). TNF-α exists in circulation as a homotrimer, approximately 52kDa in size (Smith and Baglioni, 1987). TNF-α binds to approximately 25 different receptors (Idriss and Naismith, 2000), however, the most prevalent and well characterised are TNF receptor 1 and 2 (TNFR-1/2) (Loetscher et al., 1990). TNFR-1 is fairly ubiquitously expressed across a range of cell types, whereas TNFR-2 seems to be more confined to cells of a hematopoietic origin (Ryffel and Mihatsch, 1993). Moreover, the majority of biological functions of TNF-α occur via TNFR-1 (Chen

and Goeddel, 2002). The signalling cascade initiated via TNF-α binding of TNFR-1 is very well characterised (Figure 1.3). The TNF-α homotrimer binds the TNFR-1 forming the TNF-TNFR-1 complex. The intracellular domain of the TNFR-1 is recognised and recruits TNF-receptor associated death domain (TRADD) to the complex. Additional adaptor proteins are recruited to the complex, namely receptor interacting protein (RIP) and TNF-R associated factor 2 (TRAF-2). The function of TRAF-2 is to recruit the protein cellular inhibitor of apoptosis 1 (cIAP-1) and can also activate the mitogen associate protein kinase pathway (MAPK) (Chen and Goeddel, 2002). However, RIP is a key component of TNF-α signalling by the activation of Nuclear Factor Kappa B (NFκB).

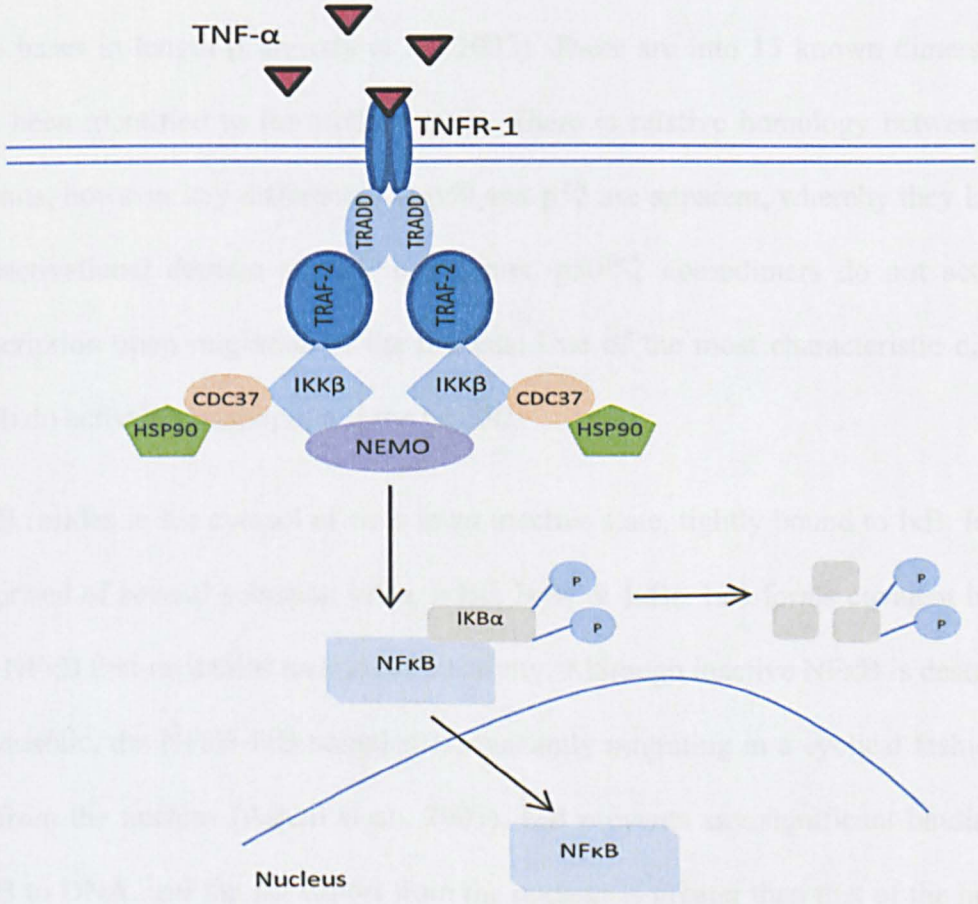


Figure 1.3 The signalling cascade initiated by the activation of TNFR-1 by TNF-α.

1.3.1 Activation of NFkB by TNF-α

NFκB is a pleitropic nuclear transcription factor comprised of 7 subunits encoded by distinct genes. The pleitropic nature of NFκB is typified by its transcription of approximately 300 genes (Ashall et al., 2009), with roles in apoptosis and cellular maturation (Perkins, 2007).

In mammalian species NFκB is comprised on the subunits p50, p52 p65 (RelA), c-Rel and RelB (Hayden and Ghosh, 2011). The individual protein subunits of NFκB bind together to form heterodimers which are defined as the NFκB complex. Dimerisation occurs at a region termed the rel-homology domain (RHD). The RHD is located on the n-terminus of each NFκB unit and is approximately 300 amino acids bases in length (Carmody et al., 2007). There are into 15 known dimers that have been identified to form NFκB units. There is relative homology between the subunits, however key differences in p50 and p52 are apparent, whereby they lack a transactivational domain at their c-terminus; p50/52 homodimers do not activate transcription upon migration to the nucleus. One of the most characteristic dimers which do activate transcription is the p65/50.

NFκB resides in the cytosol of cells in an inactive state, tightly bound to IκB. IκB is comprised of several subunits: IκBα, IκBβ, IκBγ & IκBε. IκB forms covalent bonds with NFκB that maintains its state of inactivity. Although inactive NFκB is described as cytosolic, the NFκB-IκB complex is constantly migrating in a cyclical fashion to and from the nucleus (Ashall et al., 2009). IκB prevents any significant binding of NFκB to DNA, and the net export from the nucleus is greater than that of the import – implying NFκB to be cytosolic in origin (Ashall et al., 2009).

NFκB is activated by severing of covalent bonds with IκB – this process occurs by activation of IKK. IKK is a kinase which phosphorylates IκB and initiates IκB degradation by the ubiquitin-proteasome pathway. Phosphorylation and degradation of IκB leaves NFκB free and in an active form, which is able to translocate to the nucleus, bind to DNA promoter sequences and initiate transcription. Binding of NFκB to DNA occurs in dimer form, at regions known specifically as κB domains (Hayden and Ghosh, 2011).

NFκB is activated by a variety of stimuli from viral and bacterial components to pro-inflammatory cytokines. A key activator of NFκB is TNF-α (Ashall et al., 2009). The canonical activation of NFκB due to degradation of the inhibitor of kappa B alpha/beta (IKBα/β) by IκB kinase (IKK) is TNF-dependent (Ghosh and Karin, 2002). The activation of IKKβ by TNF-α occurs due to translocation of IKKβ to the membrane by the chaperones CDC37 and HSP90; the activation of IKKβ is RIP-dependent. IKKβ phosphorylates the IKBα and IKBβ subunit which bind to and stabilise NFκB in its inactivate state in the cytoplasm. Dissociation of NFκB from its inhibitory complex allows translocation to the nucleus and activation of gene expression (Chen and Goeddel, 2002). TNF-α plays a key role in cellular signalling pathways in particular with its activation of NFκB, indicating the role of TNF-α as a master regulator of the immune response.

TNF-α is produced by a variety of cell types, such as monocytes, macrophages, NK cells, endothelial cells and smooth muscle (Idriss and Naismith, 2000). More recent research has demonstrated TNF-α expression in skeletal muscle (Plomgaard et al., 2005) and adipocytes (Hotamisligil et al., 1993).

NFκB is widely implicated in TNF-α induced loss of skeletal muscle mass (Ladner et al., 2003); activation of NFκB in muscle is associated with suppressed myogenic differentiation (Guttridge et al., 2000) and induction of the ubiquitin/proteasome pathway (Li and Reid, 2000). Moreover, activation of NFκB by TNF-α is thought to be mediated by mitochondrial ROS production, since inhibition of complex I of the electron-transport chain using rotenone showed suppressed NFκB activation (Li et al., 1999).

1.3.2 The pathological effects of TNF-α

The biological importance of TNF-α was demonstrated in several key studies throughout the 1970's and 80's. TNF-α was originally discovered over 30 years ago as a serum soluble molecule, released by macrophages, suppressed tumour growth significantly in mice (Carswell et al., 1975). In addition TNF-α was found to be the hormone named cachectin which induced cachexia in mice (Beutler et al., 1985). Treatment of rats with recombinant TNF-α induced a state of septic shock (Tracey et al., 1986). Administration of anti-TNF-α antibodies during endotoxin-induced insult provided protection against septic shock-induced cachexia, and reduced morbidity (Tracey et al., 1987). These important studies provided a key insight into the deleterious role of TNF-α during instances of profound bacterial infection and that TNF-α is likely to be a key mediator of cachexia in the critically ill. Typically during sepsis the critically ill demonstrate significantly elevated levels of TNF-α in circulation (Casey et al., 1993). Demonstration of the cachetic effect of TNF-α administration in animal models (Ling et al., 1997) suggested that TNF-α plays a significant role to play in the critically ill. Rodent models of sepsis have shown that reduced protein synthesis is associated with disrupted ribsosmal s6 kinase phosphorylation in a TNF-α-dependent manner (Lang and Frost, 2007).

1.3.3 The effect of TNF-α on myogenesis and muscle regeneration

A number of studies have examined the role that TNF-α plays in inflammatory disease states such as cancer (Tisdale, 1999) and chronic obstructive pulmonary disease (COPD), both of which demonstrate profound loss of skeletal muscle mass and function (Farber and Mannix, 2000).

There is a clear dichotomy when examining the effects of TNF-α on myogenesis. Studies examining the release of TNF-α from muscle, and the treatment of muscle with low levels indicate that TNF-α plays a pivotal role in myogenesis. Models of muscle injury demonstrate that skeletal muscle releases TNF-α, which signals in an autocrine manner to promote regeneration (Chen et al., 2005). TNF-α released as a result of muscle injury results in the phosphorylation of two key myogenic differentiation factors, myocyte enhancer factor 2 (MEF2) and E47 in a p38 MAP kinase-dependent manner (de Angelis et al., 2005). This in turn results in upregulation of MyoD, a key muscle differentiation factor (Puri et al., 2000). In contrast, other studies, have demonstrated that elevated levels of TNF-α prevents myoblast differentiation and thus suppresses the regenerative capacity of muscle (Layne and Farmer, 1999), an effect which may play a role in the overall loss of muscle mass seen with elevated circulating levels of TNF-α. Furthermore, TNF-α-induced activation of NFκB causes down-regulation of MyoD mRNA (Guttridge et al., 2000) suggesting a mechanism involved in the cachtetic loss of muscle in the critically ill. Studies have demonstrated that TNF-α also inhibits myogenesis by downregulation of the key factor Notch-1, in an NFκB-dependent manner (Acharyya et al., 2010). Overall, it is clear that low levels of TNF-α are capable of augmenting key processes in myogenesis, whereas, elevated levels as seen in the critically ill have a profound negative effect.

1.3.4 TNF-α and skeletal muscle wasting

The exposure of muscle to TNF-α results in a loss of total muscle protein, a process that is reported to be regulated by NFκB, additionally the loss of muscle protein demonstrated in this study was correlated with elevated ubquitin conjugation and augmented by endogenous production of ROS (Li et al., 1998). Overexpression of the IκBα protein (which holds NFκB in its inactive state) in muscle, results in resistance to TNF-α-induced protein loss (Li and Reid, 2000). Studies examining the inhibition of NFκB activation *in vivo* demonstrated improved skeletal muscle regeneration following trauma (Thaloor et al., 1999). Thus, there is a clear association between NFκB activation and muscle wasting during inflammation.

Although the cachectic loss of muscle protein as a result of TNF-α exposure is profound, it has been reported that the loss of muscle protein is superseded by a significant fall in specific force generation by muscle (Hardin et al., 2008). Specific force is defined as force generated by muscle per cross-sectional area. Studies into muscle contractility in the diaphragm consistently report fall in specific force generation in response to elevate levels of TNF-α (Wilcox et al., 1996). Moreover this occurrence has been reported in the absence of muscle wasting (Li et al., 2000b). Further studies have demonstrated loss of muscle function in the absence of atrophy, via TNF-α induced activation of caspase-3 which may be due to the loss of the actin and myosin contractile filaments (Supinski and Callahan, 2006).

Studies have reported that TNF-α induced loss of muscle protein occurs via the disruption of the ubiquitin/proteasome pathway (Llovera et al., 1997). The ubiquitin/proteasome controls cellular proteolytic degradation of ubiquinated proteins (Lecker et al., 1999). TNF-α administration induces elevation in ubiqutin

expression and up-regulation of the degradation pathway (Llovera et al., 1997). Up-regulation of ubiquitin-conjugating activity in skeletal muscle has been reported to be TNF-α/NFκB dependent (Li et al., 2003b). Disruption of the ubiquitin/proteasome pathway in the critically ill is the key determinant in cachectic muscle loss.

1.3.5 Role of TNF-α mediated changes in ROS in the loss of skeletal muscle mass and function

The loss of muscle mass and significant reduction in muscle force as a result of TNF-α exposure has been widely described to be associated with elevated production of ROS (Li et al., 1998). Using a rodent model of TNF-α induced cachexia, muscle loss was found to be ablated following pre-treatment with nitro-L-arginine, a known nitric oxide synthase (NOS) inhibitor (Buck and Chojkier, 1996). The up-regulation of NFκB by TNF-α in skeletal muscle is reported to be controlled by the glutathione pathway; suppression of glutathione reductase activity reduced TNF-α-induced NFκB activation (Sen et al., 1997). More recently, treatment of muscle fibres with the antioxidant Trolox (a vitamin-E derivative) resulted in attenuation in the TNF-α induced fall in specific force generation by muscle (Hardin et al., 2008). Moreover, the specific effect of ROS on muscle wasting has been investigated widely. Treatment of C2C12 myotubes with hydrogen peroxide (H_2O_2) resulted in the upregulation of the expression of ubiquitin ligases responsible for controlling protein degradation via the proteasome (Li et al., 2003a). ROS-mediated muscle proteolysis has also been associated with Ca^{2+} calpain activity. Elevated formation of reactive aldehyde complexes by ROS causes accumulation of Ca^{2+} in the cytosol, due to disruption of Ca^{2+} transport across the plasma membrane (Siems et al., 2003). Thus, inducing calpain-mediated cleavage of key proteins such as titin and nebulin, which are components of the contractile architecture (Purintrapiban et al., 2003). Although

the effect of ROS on skeletal muscle is profound it is still unclear whether elevated ROS forms part of a downstream signalling cascade which mediates muscle atrophy. Overall the research carried out on the effect of TNF-α on skeletal muscle and the subsequent loss of muscle mass and function is summarised in Figure 1.4.

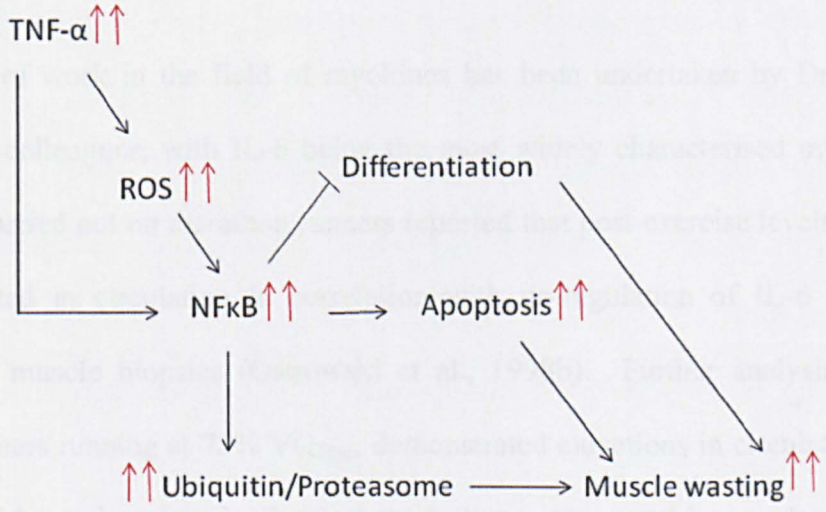


Figure 1.4 A schematic representation of the effects of TNF-α on skeletal muscle.

1.4 Muscle-derived cytokines (myokines)

The role of skeletal muscle as a locomotive organ is well defined, and has been a primary focus for muscle research for many years. However, pioneering work in the late 1990's demonstrated that skeletal muscle is not a bystander in the immune response, but is able to produce and release a number of cytokines. Myokines was the name coined to describe muscle-derived cytokines. The term myokine not only encompasses the release of cytokines, but more broadly the release of proteins from muscle, which have signalling properties to other cells or tissues.

Overall, the field of myokines is in its infancy; however there is a real prospect that skeletal muscle could be the largest cytokine contributor in the human body. Studies

have demonstrated the expression of an array of cytokines in skeletal muscle (Alvarez et al., 2002a, Bhatnagar et al., 2010). Moreover, investigations in rodents treated with LPS demonstrated elevated cytokines in both the circulation and interstitial fluid (ISF) surrounding skeletal muscle (Borge et al., 2009).

1.4.1 Interleukin-6 (IL-6)

The majority of work in the field of myokines has been undertaken by Dr Bente Pedersen and colleagues, with IL-6 being the most widely characterised myokine. Initial work carried out on marathon runners reported that post-exercise levels of IL-6 were elevated in circulation in correlation with up-regulation of IL-6 mRNA expression in muscle biopsies (Ostrowski et al., 1998b). Further analysis using human volunteers running at 75% VO_{2max} demonstrated elevations in circulating IL-6 occurring without elevations in classical pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8 (Ostrowski et al., 1998a). It was initially reported that this elevation was in response to muscle damage, whereby studies using lengthening contractions showed that elevated plasma IL-6 correlated with muscle damage (Bruunsgaard et al., 1997) Further studies have demonstrated that elevations in plasma IL-6 are dependent on high-intensity exercise, with elevations following endurance regimes only occurring in the late stages (Nielsen et al., 1996, Ostrowski et al., 2000). Initially the source of IL-6 was believed to be circulating monocytes in response to muscle damage (Nehlsen-Cannarella et al., 1997). However, examination of IL-6 mRNA in monocytes showed no changes in response to exercise, suggesting monocytes are not the source of the increased circulating IL-6 (Pedersen and Hoffman-Goetz, 2000). In addition, studies using fluorescent flow cytometry, demonstrated that IL-6 protein expression did not change in monocytes following exercise, again suggesting that these cells were not the source of elevated circulating

IL-6 (Starkie et al., 2000). Further studies examining blood from the femoral vein and biopsies from the *vastus lateralis* post-exercise reported increases in muscle IL-6 and TNF-α mRNA, but only elevation of IL-6 in the plasma from the femoral vein, indicating skeletal muscle is a source of IL-6 (Steensberg et al., 2002). A study using a rodent model of muscle contraction demonstrated that using an electrically stimulated lengthing and shortening contraction protocol resulted in the up-regulated expression of IL-6 mRNA in skeletal muscle compared with contralateral controls (Jonsdottir et al., 2000). The up-regulation of IL-6 mRNA and increased IL-6 levels in the circulation following exercise occurred in tandem with elevations in anti-inflammatory components, independent of changes in TNF-α (Steensberg et al., 2003). This was the first description that exercise could invoke an anti-inflammatory response (Figure 1.5). The anti-inflammatory effect of IL-6 was demonstrated further, whereby infusion of recombinant IL-6 suppressed endotoxin-induced elevations in TNF-α (Starkie et al., 2003).

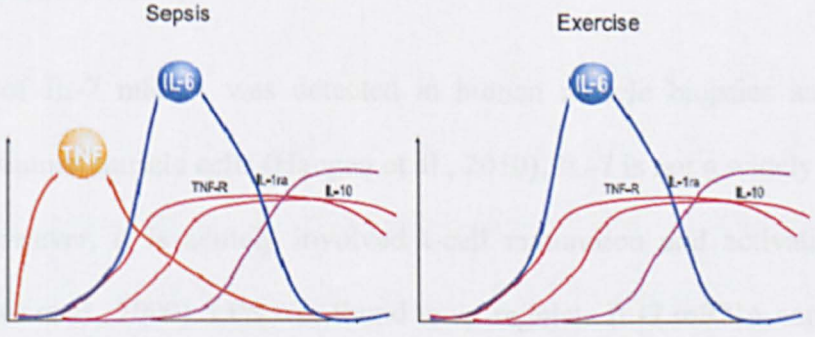


Figure 1.5 Taken from Pedersen and Febbraio (2008) A representation of the anti-inflammatory effect of exercise; inducing cytokine up-regulation independent of TNF-α.

Although skeletal muscle is the largest protein source in the human body and thus may contribute significantly to elevated levels of circulating IL-6 post-exercise, the release of IL-6 from other tissues, in particular adipose tissue, has also been

described (Keller et al., 2003a). The release of cytokines from adipocytes has been widely characterised with TNF-α being the first described adipokine (Hotamisligil et al., 1993). The area of cross-talk between muscle and fat is one of great interest, with suggestions that adipose tissue-derived TNF-α may be the "bad-driver" and that exercise induced release of cytokines from muscle can help to dampen the pro-inflammatory environment. There are common factors released from both skeletal muscle and adipose tissue e.g. IL-6, all likely to be involved in numerous endocrine and paracrine signalling cascades (Trayhurn et al., 2011). The proposal that muscle-derived IL-6 promotes a beneficial inflammatory state, whereas elevated levels of IL-6 from other sources are associated with muscle wasting/weakness (Ferrucci et al., 2002) and impaired insulin signalling and glucose uptake (Franckhauser et al., 2008) is somewhat paradoxical. It is suggested that the most beneficial effect of IL-6 is through acute elevations (via exercise), compared with the deleterious effects of chronic exposure (Pedersen and Febbraio, 2008).

1.4.2 Interleukin-7 (IL-7)

Expression of IL-7 mRNA was detected in human muscle biopsies and IL-7 is released by human muscle cells (Haugen et al., 2010). IL-7 is not a widely described cytokine; however, it is acutely involved t-cell maturation and activation in the thymus (Sawa et al., 2009). LPS was found to up-regulate IL-7 mRNA expression in skeletal muscle, in addition, maturation of human myoblasts to myotubes correlated with increased secretion of IL-7. These observations of IL-7, both *in vitro* and *in vivo*, suggest that skeletal muscle-derived IL-7 may have an important role in regulating t-cell function, which may have an impact on the systemic response to infection or insult.

1.4.3 Interleukin-8 (IL-8)

IL-8 is a potent neutrophil chemoattractant, and a key player in the innate immune response. In response to exercise, femoral artery concentrations of IL-8 were elevated, with no change in overall circulating levels, suggesting IL-8 has local angiogenic role within muscle, independent on neutrophil accumulation (Akerstrom et al., 2005). This hypothesis is supported by increased expressed of the IL-8 receptor CXCR2 in skeletal muscle following exercise (Frydelund-Larsen et al., 2007).

1.4.4 Interleukin-15 (IL-15)

IL-15 is highly expressed in skeletal muscle; *in vitro* studies reported the anabolic effect of IL-15 on muscle (Quinn et al., 2002). However, the most potent effect of IL-15 has been demonstrated *in vivo*. The anabolic effect of IL-15 has further been described in a rodent model of cachexia, whereby administration of IL-15 significantly reduced loss of muscle mass and protein (Figueras et al., 2004).

Administration of recombinant IL-15 in a rodent model induced a 33% reduction in white adipose tissue deposition (Carbo et al., 2001). More recently, elevated mRNA levels of IL-15 were detected in skeletal muscle following strength training regimes (Nielsen et al., 2007). In addition elevated circulating levels of IL-15 in rodents have been associated with reduced visceral fat and increased bone mineral density. Administration of IL-15 to dystrophic *mdx* mice improved muscle function in the diaphragm of these animals (Harcourt et al., 2005).

1.5 Heat Shock Proteins

Heat Shock Proteins (HSPs) are a family of proteins which are highly conserved across species. HSPs are classified in accordance to their molecular weight. The HSP family is organised in accordance with their monomeric molecular weight. Examples of such sub-families are HSP10, HSP60, HSP70, and HSP90; each subset has been found to harbour its own unique function, however the cytoprotective role seems to be a function conserved throughout all.

HSPs were initially discovered during studies of *drosophila melongaster* whereby elevations in temperature (heat shock) resulted in elevations in specific RNA species, in time, which was reported at the up-regulation of protein as a result of heat (Ritossa, 1962). Under quiescent conditions some HSPs are involved in binding to and stabilising nascent or newly synthesised peptides, where they are classified as molecular chaperones(Vabulas et al., 2010). The stabilisation of peptides is pivotal in the prevention of irreversible protein aggregation. Aggregation of proteins has serious pathological implications, resulting in either impaired or novel protein function. A typical example of the pathological effects of protein aggregation is demonstrated in Alzheimer's disease (AD). Aggregation of the protein β-amyloid and tau result in formation of amyloid plaques within the neural tissue, which is believed to cause the characteristic atrophy of neurons in patients with AD, thus prevention of protein aggregation has been suggested by targeting HSPs therapeutically (Wenk, 2006). Protein folding *in vitro* and *in vivo* can occur naturally, however, studies have indicated that the conditions for such spontaneity are highly dependent on factors such as overall protein concentration and temperature and pH (Fink, 1999). Typically the cell environment is a crowded place, rich in protein, metabolites and numerous organelles and cellular compartments.

HSPs prevent protein misfolding or pathological aggregation(Hartl and Hayer-Hartl, 2009).

HSP expression is up-regulated by numerous different conditions of cellular stress not only "heat shock". These include, physical, pharmacological and environmental (Hartl, 1996). Under conditions of stress HSPs levels are elevated and they bind to and stabilise proteins in an effort to promote cell survival(Morimoto, 1993). The binding of HSPs to and stabilising of peptides is a highly specific process, in nearly all cases the mechanism is ATP-dependent. Typically, a hydrophilic domain HSPs bind to a hydrophobic region of an unfolded peptide in an ATP-dependent manner to promote correct conformational folding and release of the functional peptide. A brief summary of the HSP family is shown in Table 1.1.

1.5.1 The Heat Shock Factor (HSF) family

The expression of HSPs is tightly controlled by Heat Shock Factors (HSFs) and the heat shock element (HSE). There are four HSFs that regulate transcription; HSF-1 and HSF-2 are expressed in all species of vertebrates, whereas HSF-3 and HSF-4 are limited to avians and mammals respectively (Fujimoto et al., 2004).

The HSE is a specific nucleotide sequence located near the 5' end of each HSP encoding gene. HSF-1 exists as a monomer in the cytosol under quiescent conditions it is held in its inactive state by the binding of HSP90(Buchner, 1999). Cellular stress induces release of HSP90 and migration of HSF-1 to the nucleus where it forms a homotrimer and binds to the HSE sequence on the gene of interest (Rabindran et al., 1991).

It is important to note that the increase cellular content of HSPs may not necessarily be due to a direct increase in the rate of HSP transcription. Instead the stimulus may be acting at points further upstream or downstream in the cycle such as increasing the stability of the HSP mRNA, or the efficiency of mRNA translation.

1.5.2 Heat Shock Protein 10 (HSP10/CPN10)

HSP10 is a small 10kDa nuclear encoded HSP, primarily located and functioning in the mitochondria. Encoded on human chromosome 2q33.1, HSP10 shares the same chromosome as its co-chaperonin HSP60 (Jia, 2011). The close locus of the HSP10 and HSP60 genes has been associated with simultaneous up-regulation of both genes during instances of cellular stress (Jia, 2011). In the mitochondria HSP10 and HSP60 form the chaperonin complex. The chaperonin complex binds and stabilises newly synthesised proteins in the mitochondria and this complex is pivotal in maintaining mitochondrial homeostasis. Studies have indicated HSP10 plays a significant role during cellular stress. Up-regulation of HSP10 is associated with improved outcome in ischaemia injury in cardiac tissue (Dillmann, 1999). Research from our laboratory has identified a significant role for HSP10 in protection against age-related loss of muscle mass and function, whereby overexpression of HSP10 prevented the age-related loss of force generation and muscle fibre CSA (Kayani et al., 2010).

1.5.3 Heat Shock Protein 60 (HSP60)

HSP60 (termed GroEL in bacteria) is a 60kDa protein nuclear encoded,located abundantly in the mitochondrial matrix. HSP60 is synthesised within the cell cytosol, post-translational and translocated from to the mitochondria due to the post-translational addition of a mitochondrial leader sequence (Parcellier et al., 2003). The main role of HSP60 as a mitochondrial HSP is in assisting the folding and

conformation of newly synthesised proteins and those trafficked into the mitochondria (Cheng et al., 1989). HSP60 exists as a monomer and oligomerises to form two heptameric ring structures (Hartl, 1996). The dual heptameric ring structure of HSP60 binds with HSP10 to form the chaperonin complex. The chaperonin complex is responsible for the majority of protein folding within the mitochondria. The hydrophobic lumen of the barrel-like structure contains the ATP binding domain; activation of the chaperonin complex by ATP exposes hydrophilic domains on the structure, allowing unfolded proteins to bind readily. HSP10 acts as a cap to close the top of the barrel structure to allow the chaperonin complex to successful augment correct protein folding (Cheng et al., 1989). The chaperonin complex not only folds proteins into the correct conformation it also holds certain structures in an unfolded state to allow translocation across membranes to different compartments within the mitochondria (Fink, 1999). HSP60 has been identified as a controller of apoptosis, through binding and stabilising factors involved in the apoptotic cascade (Ghosh et al., 2008).

1.5.4 Heat Shock Protein 70 family

The HSP70 family is perhaps the most widely investigated HSP subset. All HSPs are stress-inducible by definition but some are also constitutively expressed. The family comprises HSP70 which is highly stress inducible, HSC70 a constitutively expressed but inducible family member and GRP75/78 which are glucose sensitive stress proteins (Kiang and Tsokos, 1998). HSP70 has a bacterial homologue, present in *e.coli* termed DnaK (Bardwell and Craig, 1984).

HSP70 is up-regulated in response to many factors, such as mechanical and bacterial stress, as well as in response to increased cytokines, ROS and pharmacological

agents (Lee et al., 2007). Following cellular stress HSP70 can account for approximately 20% of total cellular protein (Donati et al., 1990).

Nuclear encoded HSP70 functions primarily within the cytosol. HSP70 contains a C-terminal peptide binding region and an N-terminal ATP binding region (Liu and Steinacker, 2001). HSP70 is bound in an inactive state in the cytosol to ATP, amino acid sequences are recognised at the peptide binding region, hydrolysing ATP allowing HSP70 to freely bind and stabilise the target peptide (Flaherty et al., 1990). Primarily, HSP70 preserves the structure of newly synthesised proteins and ensures correct folding during synthesis and trans-membrane trafficking of those proteins to other cellular compartments (Schmitt et al., 2007). The stress inducible form of HSP70 has been described to have significant anti-apoptotic properties (Beere et al., 2000). Moreover, research has demonstrated HSP70 expression is a key determinant of mortality in the elderly during sepsis whereby aged HSP70 knockout mice showed higher rates of mortality and exacerbated inflammatory response in a CLP model of sepsis (McConnell et al., 2011).

Studies examining the effect of overexpression of HSP70 in macrophages carried out by transfection demonstrated that HSP70 inhibited inflammatory cytokine production induced by bacterial (LPS) infection, a process mediated by NFκB (Shi et al., 2006). NFκB is one of the most prominent cytokine transcription factors in the inflammatory response; therefore it can be suggested that intracellular HSP70 has an anti-inflammatory capacity by interaction with NFκB (Chen et al., 2004).

HSC70 is a member of the HSP70 sub-family of heat shock proteins. HSC70 is a widely abundant chaperone comprising approximately 3% of total cellular protein. HSC70 is constitutively expressed, nuclear encoded and resides within the cytosol. A

characteristic of the majority of HSP family members is their induction during instances of stress. However, HSC70 is widely described as the least reactive, and generally is not poorly up-regulated in response to stimuli (Callahan et al., 2002). In similar manner to other family members HSC70 has a role in stabilising newly synthesised peptides in an ATP-dependent manner. Furthermore, cellular stress results in migration of HSC70 to the nucleus to bind unstable or aggregated proteins, where it also interacts with HSF to shut down the stress response (Liu and Steinacker, 2001).

1.5.5 Heat Shock Protein 90 (HSP90)

HSP90 is one of the largest subsets of HSP at 90kDa and accounts for approximately 1% of total cellular protein in quiescent conditions (Welch and Feramisco, 1982). The HSP90 family of proteins demonstrates cytoprotective function in a similar manner to the HSP70 family. HSP90 is present in large quantities within the cytosol of cells in two different isoforms, HSP90α and HSP90β. GP96 is another member of the HSP90 sub-family. GP96 is an endoplasmic reticulum associated molecule (Broquet et al., 2003). HSP90 follows the ATP dependent mechanism of protein stabilisation, whereby upon binding the ATP the HSP90 changes in conformation to allow interaction with mis-folded or aggregated proteins. However in similar manner to HSP70, HSP90 also inhibits the apoptosis cascade at different points to prevent cell death under conditions of stress, by binding to and stabilising caspase proteins (Li et al., 2000a). HSP90 plays a significant role in cellular function due to the large repertoire of client proteins that it binds – approximately 60-70 in total. The majority of research into HSP90 is in the pathology of cancer, whereby HSP90 is implicated in binding and maintaining mutant forms of p53 – a tumour suppressor protein (Picard, 2002). HSP90 retains HSF-1 in an inactive state; inhibitors of HSP90 such

as 17AAG have been shown to up-regulate HSP expression, due to the release of HSF-1 from its inactive state (Kayani et al., 2008).

1.5.6 Classical role of HSPs in skeletal muscle

Several studies have investigated the cytoprotective function of HSPs in skeletal muscle, in an effort to find an avenue of therapeutic intervention. Elevation in HSP27, HSP60 and HSP700 content is seen in the muscle of humans following exercise training (Morton et al., 2009) and following acute non-damaging contraction protocols in humans (Khassaf et al., 2001, Morton et al., 2006) and in rodents (McArdle et al, 2001) Furthermore, cellular stress analogous to muscle damage *in vivo* resulted in elevated HSP60 content of C2C12 myotubes *in vitro* (Maglara et al., 2003). Overall, HSP60 is sensitive to damage and cellular stress and is a key component in cytoprotection.

The cytoprotective role of HSP70 has been demonstrated in muscle. Overexpression of HSP70 provided considerable protection against contraction-induced damage in muscles of adult and old mice and reversed the inability of muscles of old mice to regenerate following damage (McArdle et al., 2004). Moreover, this preservation in muscles of old mice was associated with normalisation of ROS production and correction of aberrant NFκB activation in old muscles (Broome et al., 2006). Acute elevation of HSP70 content of muscle *in vivo* using the HSP90 inhibitor, 17-(allylamino)-17-demethoxygeldanamycin, also enhanced the recovery of muscle of old mice following a damaging contraction protocol (Kayani et al., 2008). Studies from our laboratory examining the effect of overexpression of HSP10 in skeletal muscle have also demonstrated the cytoprotective effect of HSP10 in muscle.

Overexpression of HSP10 in skeletal muscle protected against the age-related loss of specific force and fall in fibre cross-sectional area (Kayani et al., 2010).

HSP	Location	Reference.
αB-crystallin	**Cytosol**	**(Fink, 1999)**
HSP10	**Mitochondria**	**(Kayani et al., 2010)**
HSP25/27	**Cytosol, nuclear**	**(Fink, 1999)**
HSP60	**Mitochondria, cytosol**	**(Hartl, 1996)**
HSP70	**Cytosol**	**(McArdle et al., 2004, Ritossa, 1962)**
HSP90	**Cytosol**	**(Buchner, 1999)**

Table 1.1 A brief summary of HSPs and their cellular locations.

1.6 Extracellular HSPs (eHSPs)

In recent years increasing evidence has demonstrated the presence of HSPs in the extracellular environment (Pockley and Multhoff, 2008). The functions of eHSPs have been increasingly investigated over recent years, with immuno-stimulatory properties being a common function (Radons and Multhoff, 2005). Several studies have proposed a dual function of HSPs both as chaperones but also with chemokine-like properties (Asea, 2008b). The difference between how HSPs function in an intracellular or extracellular environment is fascinating. Whether this is directly as a result of the environment or perhaps conformational differences in HSP structure invoking these different functions is unknown. For example, subtle changes such as pH in the extracellular environment may alter the tertiary structure of HSPs, which in turn may render them to act in a more cytokine-like manner, however, this concept is poorly understood.

The first description of HSPs located in the extracellular environment was made in the mid 1980's, whereby HSP transfer was shown from glia to axon (Tytell et al., 1986). However, the demonstration of HSP70release from mammalian cells by non-golgi mediated means was perhaps the first suggestion that HSP release may in-fact be selective and have functional consequences (Hightower and Guidon, 1989). The release of a leaderless HSP70 protein from viable cells is intriguing but the functional significance of eHSPs was not realised until over a decade later. Overall eHSPs have been described as danger-signals which can prime an immune response in light of an impending stress or insult (Multhoff, 2007).

1.6.1 Extracellular HSP10 (eHSP10)

The function of extracellular HSP10/CPN10 is poorly understood. eHSP10 was first identified as early pregnancy factor (EPF)– whereby elevated levels of EPF was correlated with immunosuppresssion during preganacy (Noonan et al., 1979). EPF was not identified as HSP10 until 20 years later (Cavanagh and Morton, 1994). HSP10 is elevated in the circulation of individuals with periodontal disease (Shamaei-Tousi et al., 2007a). Moreover, elevated circulating HSP10 was found in patients with ovarian cancer (Akyol et al., 2006) and *in vitro* studies have demonstrated that HSP10 is released from human umblicial vein endothelial cell (HUVECs) following treatment with erythropoetin (Dobocan et al., 2009).

Studies have demonstrated widespread anti-inflammatory effects of eHSP10. Treatment of macrophages with HSP10 resulted in suppressed release of RANTES and IL-6 following LPS stimulation (Johnson et al., 2005). Administration of recombinant HSP10 to patients with rheumatoid arthritis resulted in marked improvement in their condition (Vanags et al., 2006) and treatment of patients with

multiple sclerosis with recombinant HSP10 (Broadley et al., 2009) resulted in clinical improvement. However, the precise mechanism of action of eHSP10 remains poorly understood.

1.6.2 Extracellular HSP60 (eHSP60)

HSP60 has been widely described as a mitochondria-associated protein. HSP60 is nuclear encoded, and translocated into the mitochondria (Parcellier *et al.* 2003) where it functions as part of the chaperonin complex with HSP10. In contrast to HSP10, studies have indicated that HSP60 is primarily pro-inflammatory in the extracellular environment where it is associated with a variety of inflammatory disorders (Pockley and Multhoff, 2008).

1.6.3 The presence of eHSP60 in disease states.

The first description of HSP60 in the circulation was made during studies examining serum from healthy individuals. HSP60 is abundant in the circulation, with females having approximately 3-fold higher levels than that of males, however, no clinical relevance has been identified from this observation (Pockley et al., 1999). Elevated serum levels of HSP60 have been correlated to atherosclerosis (Xiao et al., 2005), hypertension, cardiovascular disease (Pockley et al., 2000) and a higher risk of coronary heart disease (Zhang et al., 2008). A larger cohort study examining ~10,000 individuals demonstrated the presence of HSP60 in the circulation of healthy individuals. Approximately 50% of individuals had detectable levels of HSP60 in serum, with a suggestion that baseline serum levels are determined by genetic predisposition (Shamaei-Tousi et al., 2007b). A positive correlation was found between serum HSP60, cardiovascular disease and diabetes (Shamaei-Tousi et al., 2006). Studies examining patients with type II diabetes reported that levels of

HSP60 in serum correlated with HSP60 levels detected in saliva, and the authors suggested that HSP60 could be used as a biomarker of the disease (Yuan et al., 2011). Overall, these studies suggest an association of elevated serum levels of HSP60 with a stress or disease state.

Although HSP60 is primarily located in the mitochondria, more recent evidence suggests that a population of HSP60 that resides on/within the plasma membrane of cells such as cardiac myocytes (Lin et al., 2007). It has been suggested that this population may be the source of eHSP60 (Gupta and Knowlton, 2007). Extracellular HSP60 is elevated in septic patients and it is suggestedthat HSP60 may contribute to the pathogenesis of sepsis and the overall hyperinflammatory state in this condition (Wheeler *et al.* 2006). In patients with acute lung injury the progression of the trauma has been correlated with elevated serum levels of HSP60 (Pespeni *et al.* 2005). Additional research has reported that HSP60 is selectively secreted from tumour cells (Merendino et al., 2010).

1.6.4 The immunomodulatory function of eHSP60

Evidence suggests that eHSP60 functions in an immunostimulatory manner. The first study to examine the immunostimulatory function of eHSP60 demonstrated HSP60 stimulated cytokine release from peripheral blood mononuclear cells (PBMCs) in a CD14-dependent manner; CD14 is the co-receptor for TLR-4 (Kol et al., 2000).

In contrast, other studies have defined eHSP60 to signal in an anti-inflammatory manner, capability of interacting with T-Cells via TLR-2 resulting in down-regulation of chemokine receptor expression upon the cell surface, and prevention of T-Cell chemotaxis (Zanin-Zhorov et al., 2003). This interaction has been investigated further; describing HSP60 as a co-stimulator of CD4+ and CD25+ T-

regulator cells again via TLR-2 (Zanin-Zhorov et al., 2006). Furthermore, HSP60 has been found to activate B-cells through TLR-4 (Cohen-Sfady et al., 2005). Additional studies have demonstrated that HSP60 stimulates monocytes to release IL-8 via TLR-2 (Zhao et al., 2007). Study examining soft tissue trauma using a hip replacement model, showed release of HSP60 into wound drains which then demonstrated an immunosuppressive function upon monocytes – dampening LPS-induced TNF-α release in a HSP60-dependent manner (Flohe et al., 2007). Studies have also demonstrated that eHSP60 can drive bone resorption, which has implications for frailty in the elderly (Koh et al., 2009).

1.6.5 Extracellular HSP70 (eHSP70)

The first description of HSP70 in the extracellular environment was demonstrated using rat embryo cells (Hightower and Guidon, 1989). A decade later and the functional significance of eHSP70 were demonstrated, whereby eHSP70 stimulated cytokine production in an NFκB dependent manner from human monocytes (Asea et al., 2000b). This study was the defining moment in the dual-function of HSPs.. Historically a mantra of "one gene, one protein, one function" had been widely accepted, however, the dual-functions of proteins provided a new and intriguing insight into the way we examine cellular signalling as a whole. Hence, eHSP70 was the first HSP to be identified as a chaperokine – demonstrating both chaperone and cytokine-like properties (Asea, 2008b).

1.6.6 The immunomodulatory functions of eHSP70

The majority of literature indicates that eHSP70 is pro-inflammatory. In addition to up-regulating cytokine production (Asea et al., 2000b), eHSP70 up-regulates inducible nitric oxide synthase (iNOS) in and increases nitric oxide release from

antigen presenting cells (Panjwani et al., 2002). The first demonstration of HSP70 presence in the serum under pathological conditions was in peripheral and vascular renal disease (P/RVD) whereby elevated levels of eHSP70 correlated with presentation of PVD and RVD in patients, compared with healthy controls (Wright et al., 2000).

The signalling capability of eHSP70 is augmented through the TLR family of receptors, in particular via a TLR-4 and CD14 mediated mechanism (Vabulas et al., 2002). Studies further identified TLR-4 as a primary receptor for eHSP70 and it augments cytokine production through activation of NFκB in airway epithelial cells (Chase et al., 2007). This work was reinforced with the description of eHSP70 as an endogenous damage-associated molecular pattern (DAMP), with eHSP70 released from airway epithelial cells and capable of activating neutrophils, key mediators of the inflammatory response in respiratory pathologies (Wheeler et al., 2009).

1.6.7 The presence of eHSP70 in disease states

eHSP70 is readily detected within the serum, with an inverse correlation to the severity of cardiovascular disease (Zhu et al., 2003, Pockley et al., 2003). This observation is somewhat paradoxical to the described pro-inflammatory function of eHSP70.

In addition, eHSP70 derived from mycobacterium, provokes cytokine up-regulation via the CD40 receptor from human monocytes (Wang et al., 2001). Moreover, research has indicated that eHSP70 is a ligand for CC chemokine receptor 5 (CCR5) suggesting that eHSP70 may play a role in the pathogenesis of chronic inflammatory diseases such as inflammatory bowel disease (Whittall et al., 2006). More recently research has indicated eHSP70 can bind to the receptor CD91 and activate $CD4^+$ T-

cells (Fischer et al., 2010). Overall, the receptor family for eHSPs is poorly understood. However, a unilateral characteristic is the involvement of the TLR family of proteins.

1.6.8 Mechanisms of release of HSPs into the extracellular environment

HSP family members lack a classical leader sequence and therefore lack the ability to be secreted via golgi-mediated apparatus. Studies have demonstrated that are HSPs released either passively by cellular necrosis, or actively, by non-classical means from viable cells (Clayton et al., 2005). HSP70 has been found in exosomes, nano-sized vesicles of endocytic origin (Bausero et al., 2005, Gastpar et al., 2005, Zhan et al., 2009). The biogenesis of exosomes involves the recycling of plasma membrane components into the exosome lumen (Johnstone, 2006). It is suggested that a large proportion of HSP60 and HSP70 released into the extracellular space is through the exosomal pathway (Gupta and Knowlton, 2007).

1.7 Exosomes

Exosomes are small nano-sized vesicles, 50-90nm in diameter, of endocytic origin (Johnstone, 2006). Studies examining the removal of the transferrin receptor from reticulocytes during maturation to erythrocytes demonstrated that the receptor was released as part of membranous vesicles (Harding et al., 1983, Johnstone et al., 1984, Johnstone et al., 1989). The biogenesis of exosomes has been studied extensively to determine the precise inter-relationship of exosomes with golgi-lysosome network.

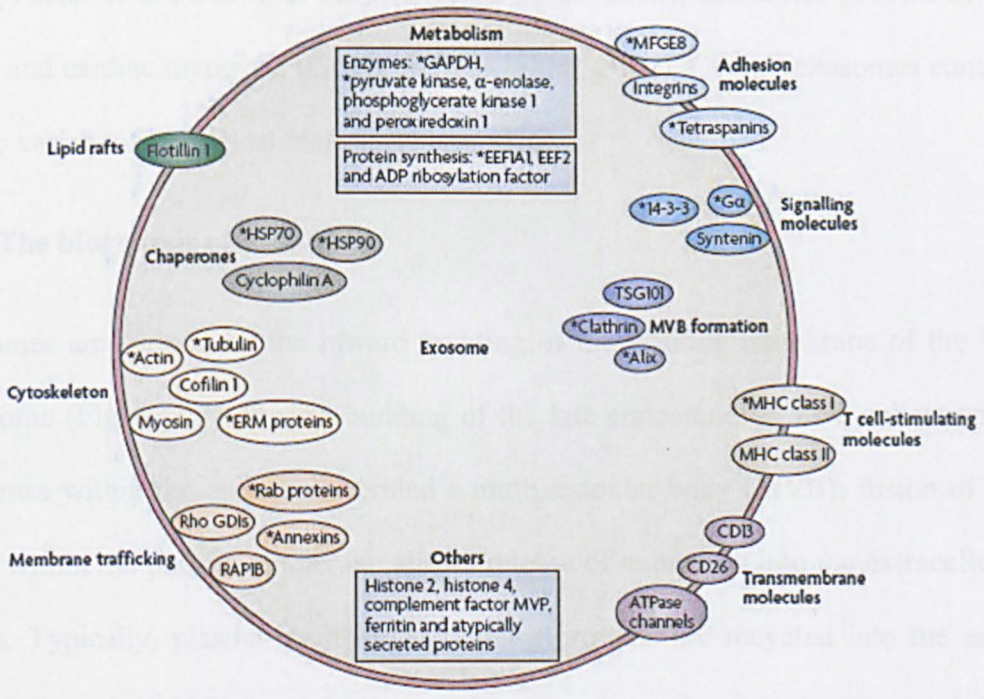


Figure 1.6 Canonical exosome structure (taken from Thery et al. 2009)

Exosomes are homogenous across cell types, with an outer membrane analogous to the plasma membrane of the originating cell. Exosome size (50-90nm) and density (1.1-1.3g/ml) are features which make exosomes distinct from particles shed by plasma membrane blebbing (Valadi et al., 2007). Thus, exosomes are highly enriched with membrane spanning proteins, in particular the tetraspanins CD9, CD63 and CD81 (Eldh et al., 2010). Exosomes typically display proteins from their cell of origin on the outer membrane, such as MHC class II on T-cell derived exosomes (Buschow et al., 2009). The contents of the exosomal lumen is highly heterogenous, also dependent on the cells of origin, however there are a few common features between exosomes from different cell types. Expression of HSPs, in particularly HSC70 (Lasser et al., 2011) and the proteins TSG-101 and Alix (Guescini et al., 2010) in the lumen of exosomes is widely described – such that these proteins are used as markers of exosomes. HSPs have been identified in exosomes from mast

cells (Valadi et al., 2007), B cells (Clayton et al., 2005), astrocytes (Taylor et al., 2007) and cardiac myocytes (Gupta and Knowlton, 2007). Overall exosomes contain a wide variety of biological material (Figure 1.6).

1.7.1 The biogenesis of exosomes.

Exosomes are formed by the inward budding of the limiting membrane of the late endosome (Figure 1.7). Inward budding of the late endosome forms a collection of exosomes within the endosome termed a multivesicular body (MVB); fusion of the MVB within the plasma membrane allows release of exosomes into the extracellular milieu. Typically, plasma membrane spanning proteins are recycled into the early endosome and trafficked to the late endosome where they are incorporated into exosomes (Fevrier and Raposo, 2004). Proteins from the golgi network can also be targeted to the late endosome for either incorporation into exosome or degradation via the lysosome (Denzer et al., 2000). The precise stimuli and molecules which control exosome biogenesis are not fully understood. However, inhibition of Na^+/H^+ and Na^+/Ca^{2+} pumps and chelation of Ca^{2+} has been found to inhibit the release of exosomes (Savina et al., 2003). Recent research has demonstrated that the RAB genes which encode Rab GTPases, in particular Rab27a and Rab27b control exosome secretion (Ostrowski et al., 2010).

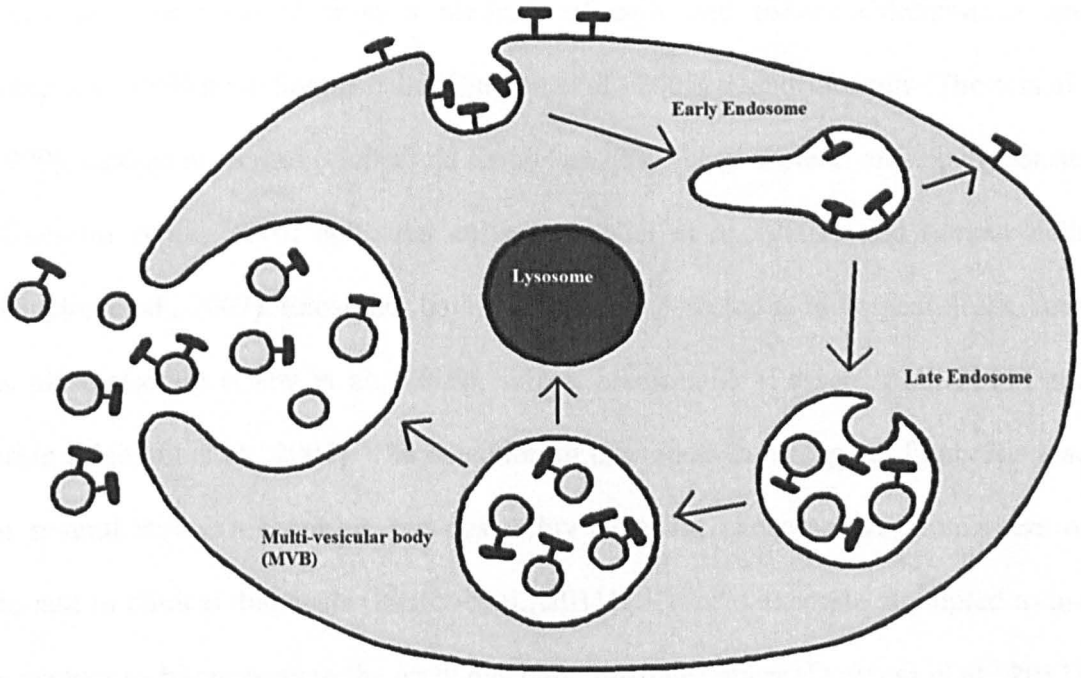


Figure 1.7 The pathway of exosome biogenesis.

The highly conserved machinery involved in exosome biogenesis across cell and tissue types suggests that almost all cell types have the capacity to secrete exosomes. Exosome release is constitutive in some cell types such as murine (mc/9) mast cells, whereas their human mast cells (HMC-1) require Ca^{2+} stimulation for exosome release (Valadi et al., 2007).

1.7.2 The biological functions of exosomes.

The initial role of exosomes was thought to be one of waste removal; however, the immunomodulatory function of exosomes has now been demonstrated. Pioneering work carried out in the mid-1990's demonstrated the release of exosomes from dendritic cells (DC) which can suppress tumour growth *in vitro* (Raposo et al., 1996, Zitvogel et al., 1998). This work suggested that exosomes have more a specific role in cellular communication rather than just as a means of cellular waste removal.

Exosomes are released from a plethora of cells and tissues (Mathivanan and Simpson, 2009) including, B cells (Clayton et al., 2005), Dendritic cells (Thery et al., 1999), cardiac myocytes (Gupta and Knowlton, 2007) and skeletal muscle myoblasts (Guescini et al., 2010) epithelial cells (van Niel et al., 2001) and tumour cells (Clayton et al., 2007). Exosomes have been readily detected in biological fluids, such as plasma/serum (Caby et al., 2005), saliva, breast milk (Lasser et al., 2011) and urine (Pisitkun et al., 2004). The detection of exosomes in biological fluids has lead to several studies examining the possibility of using exosomes as biomarkers of disease in clinical diagnosis (Keller et al., 2011). A recent example attempted to use exosomes as biomarkers in the early detection prostate cancer (Duijvesz et al., 2011). However, currently exosome isolation is a labour intensive process and no clinical significance has been correlated with proteins found enriched in exosomes.

Study of the immunostimulatory capacity of exosomes has centred primarily on T-cell function and activation. Several studies have indicated that exosomes can be derived from antigen presenting cells (APCs), exosomes enriched with MHC II on their outer membrane have been shown to activate T-cells (Admyre et al., 2006). This function is dependent on the presence of dendritic cells DC in tandem with MHC II enriched exosomes *in vitro* (Thery et al., 2002). Exosomes are released from epithelial cells of the intestine (van Niel et al., 2001) and have been shown to be immunostimulatory, upregulating humoural responses *in vivo* (Van Niel et al., 2003) Studies have also examined the use of exosomes for cancer therapy. Research has shown that tumour-derived exosomes contain antigens that can be presented to dendritic cells (DC) which activate T-cells and an anti-tumour response (Wolfers et al., 2001).

Exosomes also contain nucleic acids, specifically mRNA and microRNA, suggesting that they may be a means of genetic transfer. Exosomes are capable of transferring mRNA between cells, invoking expression of novel proteins in target cells (Valadi et al., 2007). More recently, work has demonstrated that exosomes are a significant source of micro-RNAs, small non-coding RNAs involved in post-translational modification and gene control. Moreover, exosomes containing micro-RNA are involved in regulation of the immune response, through post-transcriptional gene suppression (Zhou et al., 2011).

Recent studies have explored the use of exosomes in a therapeutic manner. The most significant advance has used exon-skipping technology. Naïve exosomes containing a specific short interfering RNA (si-RNA) can be targeted to specific tissues *in vivo* as a means of delivering gene therapy (Alvarez-Erviti et al., 2011). Although in its infancy, the use of exosomes as intercellular communicators is one of significant interest.

1.8 Muscle dysfunction and ageing: role of HSPs and a pro-inflammatory environment.

1.9.1 Skeletal muscle ageing.

As we age, our muscles demonstrate loss of mass and strength, increasing fatigability and susceptibility to injury (Marcell, 2003), and impaired capacity for recovery (Faulkner et al., 2007). By the age of 70, the cross-sectional area (CSA) of skeletal muscle is reduced by 25-30% (Porter et al., 1995) and this is accompanied by loss in absolute force (Grimby and Saltin, 1983) as well as a decrease in specific force (normalized by CSA) of the remaining muscle(Morse et al., 2005). After the age of 70, strength continues to fall 1-2% per year and power in the lower leg declines 3.5%

per year (Skelton et al., 1994). Such deficits profoundly impact quality of life for even healthy older people, as many are at or near functionally relevant strength thresholds that limit the ability to carry out everyday tasks (Young and Skelton, 1994). The mechanisms by which this age-related loss of muscle mass and function occur are unclear but our research group have hypothesised that the inability of muscles of old mammals to respond to stress is a major factor in the development of dysfunction.

1.8.2 HSPs and muscle ageing.

Skeletal muscles of adult mice and humans adapt rapidly to stress with changes in structural, metabolic, contractile, and cytoprotective proteins. Among the proteins demonstrating adaptive responses to contraction are Heat Shock Proteins (HSPs) and this response is detailed in Section 1.7. Many of these changes in gene expression are mediated and/or modulated by ROS through the activation of redox-sensitive TFs (Jackson et al., 2002). Increased extracellular levels of cytokines such as TNF-α, independent of the source cell, result in the activation of NFκB in skeletal muscle (Li et al., 1998, Sen et al., 1997) and changes in gene expression required for TNF-α-induced muscle catabolism are regulated by NFκB (Reid et al., 2011). NFκB activation is associated with increased production of pro- and anti-inflammatory cytokines and chemokines by muscle cells, which can then exert both autocrine and paracrine effects.

Muscles of old mice demonstrate chronic constitutive activation of redox-sensitive TFs (Vasilaki et al., 2006b) and an inability to further activate NFκB following a non-damaging physiological contraction protocol (Vasilaki et al., 2006a). This

inability to respond to contractions with NFκB activation is associated with a severe attenuation of normal changes in gene expression (Demirel et al., 2003, Hall et al., 2000, Heydari et al., 2000, Locke and Tanguay, 1996, Muramatsu et al., 1996, Rao et al., 1999, Vasilaki et al., 2006b). Our research group has demonstrated that the increased HSP content and antioxidant enzyme activities evident following isometric contractions in muscles of adult rodents were abolished in old rodents (Vasilaki et al., 2002, Vasilaki et al., 2006b) and this inability to adapt was associated with the lack of complete activation of the appropriate TFs (Vasilaki et al., 2006b). Studies from our laboratory also revealed that the inability to produce HSPs is associated with the functional deficits that occur in skeletal muscle with aging. Muscle CSA was preserved in old mice which were overexpressing HSP10 compared with WT mice (Kayani et al., 2010). In addition, transgenic mice that over-express HSP70 showed that an increased muscle HSP70 content provided protection from the fall in specific force associated with aging and facilitated rapid and successful regeneration following contraction-induced damage (Brooks and Faulkner, 1990, McArdle et al., 2004). This protection was associated with the prevention of oxidative damage and importantly, levels of NFκB activation at rest and following contractions similar to those in muscles of adult mice (Broome et al., 2006). These data indicate a close relationship between preservation of muscle mass and retention of the ability of muscle from old mice to regenerate and the activation status of NFκB. The mechanisms by which increased muscle content of HSP70 exert these effects on NFκB are unclear although there is evidence from other studies that HSP70 can interact directly with IKβ kinase (IKK) has been demonstrated (Chen et al., 2004, Salminen et al., 2008).

1.8.3 Cytokines, ageing and muscle.

Aging is associated with a systemic increase in inflammatory cytokines such as TNF-α (Visser et al., 2002). Baseline expression of TNF-α (Greiwe et al., 2001), IL-1β (Jozsi et al., 2001, Przybyla et al., 2006), and chemokine (C-C motif) ligand 5 (Jozsi et al., 2001) is also elevated in muscles of elderly, compared with young, subjects, but the effect of stress on these inflammatory mediators is less clear. Resistance exercise increases IL-1β gene expression in muscle of young, but not old, subjects (Jozsi et al., 2001, Przybyla et al., 2006), whereas downhill running results in 2- to 3-fold increases in TNFα and IL-1β mRNA levels in vastus lateralis muscles of old subjects, a response that varied only slightly from that observed for the young subjects (Hamada et al. 2004). The discrepancy in the results may be due to the damaging vs. non-damaging nature of the exercise, as the increases in TNF-α and IL-1β were associated with an increase, albeit blunted compared with the young subjects, in the accumulation of transcripts for the leukocyte surface marker CD18 following the injurious exercise. In contrast, resistance exercise did not alter the total number of macrophages (CD68+) nor the distribution of pro- (CD11b+) and anti-inflammatory (CD163+) populations of macrophages in the muscle (Przybyla et al., 2006). Greater accumulation of both neutrophils and macrophages has been shown in muscles of old, compared with adult, mice following a damaging protocol of lengthening contractions despite no difference between age groups in force deficits or numbers of overtly injured fibers (Koh et al., 2003). Numerous factors may play a role in the increased inflammatory cell numbers in old versus adult mice including differences in chemoattractant release, in chemotaxis to injured sites, or in the function or lifespan of the inflammatory cells.

In summary, evidence suggests that aging results in regulatory defects both locally within the muscles and systemically that alter cytokine expression and inflammatory cell function, both at rest and in response to stress. Not at all clear is the connection or directionality between systemic and muscle defects.

1.9 Summary

In summary, skeletal muscle is a pivotal organ in the critically ill, whereby loss of muscle is a predictor of mortality. The identification that skeletal muscle can act as a source of signalling factors provides a new perspective on the role of muscle in the critically ill. The release of factors which can signal in autocrine, paracrine or endocrine manners suggests that, particularly in the critically ill, muscle may contribute to its own deterioration, and multiple organ failure as a whole. However, the molecules released from skeletal muscle and the potential signalling properties of these is poorly understood and is further complicated during the ageing porcess. A better understanding of the basic mechanisms of the role of skeletal muscle as an endocrine organ is needed to dissect out avenues for therapy in such multifactorial disorders such as sepsis.

1.10 Aims

The aims of thesis are:

- Determine if skeletal muscle can act as a source of eHSPs during inflammation *in vitro*.
- Elucidate the mechanism of HSP release from skeletal muscle, by examining the exosomal pathway.

- Identify the autocrine and paracrine signalling properties of eHSPs. Signalling back to skeletal muscle and to other tissues (e.g. bone).
- Determine the effect of inflammation on the release of cytokines (myokines) from muscle.
- To determine the contribution of muscle-derived cytokines and eHSPs to the overall inflammatory state.
- To determine the impact of ageing on the ability of skeletal muscle to act as an endocrine organ.

1.11 Hypothesis

We hypothesise that:

- TNF-α induces a stress response in skeletal muscle through the elevation in intracellular HSPs and the release of HSPs into the extracellular environment.
- eHSPs are released from viable muscle by non-classical exosomal pathway.
- eHSPS and exosomes containing eHSPs can signal in both an autocrine and paracrine manner.
- Skeletal muscle acts as a significant source of myokines during systemic inflammation *in vivo*, which contribute to the overall hyperinflammatory state.
- Ageing affects the ability of muscle to act as an endocrine organ and this contributes to the aberrant inflammatory environment in the elderly.

CHAPTER 2

METHODS

2.1 Cell culture techniques and sample preparation

2.1.1 Culture of cells

- Dulbecco's Modified Eagles Medium DMEM (Sigma Aldrich, Dorset, UK).
- L-Glutamine (Lonza, UK).
- Penicillin/Streptomycin (Sigma Aldrich, Dorset, UK).
- Fetal calf serum (FCS) (Sigma Aldrich, Dorset, UK).
- Horse serum (Lonza, UK).

The C2C12 myoblast cell line, a well characterised muscle cell line, which retains almost all characteristics of *in vivo* mouse skeletal muscle (Yaffe and Saxel, 1977) and SaOs-2 osteoblast like cell line (Rodan et al., 1987) were grown in a culture medium comprising Dulbecco's Modified Eagles Medium (DMEM, Sigma, Dorset, UK), supplemented with 12% Fetal Calf Serum (FCS) (v/v) with 2mM L-glutamine, 50 i.u penicillin and 50μg/ml streptomycin. Cells were grown in a 5% CO_2 rich humidified environment at 37°C. Cells were passaged once they reached approximately 60-70% confluency. Initial cell culture was carried out in T75 (Costar, London, UK) tissue culture flasks and further sub-cultured into 6-well plates (Costar, London, UK) prior to treatment.

Myotube formation in the C2C12 cell line was induced by the replacement of the 12% FCS containing media with DMEM containing 2% Horse Serum (HS) with 2mM L-glutamine over a 5-day period. The 2% HS media encouraged the fusion of myoblasts to form characteristic myotubes (Maglara et al., 2003). Differentiation was assessed periodically throughout the 5 day period by light microscopy.

2.1.2 Treatment of cells

- TNF-α (murine) endotoxin-free (Research & Development Systems).
- Endotoxin-free HSP60 (Enzo life sciences, Exeter, UK).
- Anti-TLR2 antibody (Abcam, Cambridge, UK).
- Anti-TLR4 antibody (Abcam, Cambridge, UK).
- Brefeldin A (BFA) (Sigma Aldrich, Dorset, UK).
- Dimethyl amiloride (DMA) (Sigma Aldrich, Dorset UK).

C2C12 myotubes were exposed to either carrier-free recombinant TNF-α (10, 25 or 50ng/ml) (Guttridge et al., 2000, Langen et al., 2001), HSP60 (250, 500 or 1000ng/ml) (Shamaei-Tousi et al., 2006), anti-TLR2/4 antibodies (20μg/ml) (Kim et al., 2009), Brefeldin A (BFA) (1mg/ml) or DMA (15nM) (Gupta and Knowlton, 2007). Following treatment cell culture media was removed and stored. Cells were harvested and the cell pellet was stored for analysis by western blotting, qPCR, Luminex/ELISA. Separate cultures of cells were used for LIVE/DEAD or trypan blue viability assays as described below.

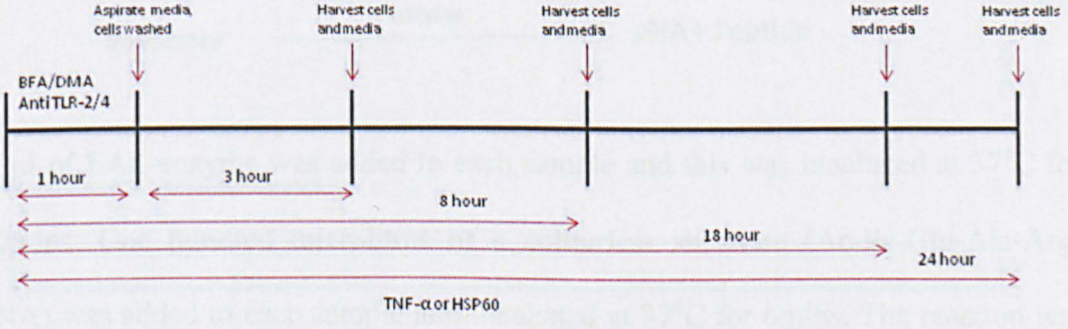


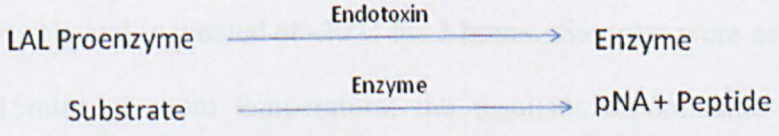
Figure 2.1 Treatments and timecourse used in studies of the effect of TNF-α and HSP60 on C2C12 myotubes.

2.1.2.1 Limulus amoebocyte lysate (LAL) assay

To determine whether the commercially obtained protein preparations contained endotoxin contaminants it was essential to determine the presence of endotoxin using a commercial LAL assay kit (Lonza, UK). The assay contained the following reagents:

- LAL proenzyme
- *Escherichia coli* stock solution
- LAL reagent water
- 10% Sodium dodecyl sulphate (SDS) (Sigma Aldrich, Dorset, UK).

The Limulus amoebocyte lysate (LAL) assay was used to determine levels of endotoxin (gram-negative) contamination in treatment preparations and media. The principle of the assay is shown below. Presence of any endotoxin converts the LAL proenzyme into its active form. The active form of the enzyme cleaves the pNA from the (Ac-lle-Glu-Ala-Arg-pNA) peptide producing a colorimetric change.



50µl of LAL enzyme was added to each sample and this was incubated at 37°C for 10mins. One hundred microlitres of a colourless substrate (Ac-lle-Glu-Ala-Arg-pNA) was added to each sample and incubated at 37°C for 6mins. The reaction was stopped by the addition of 100µl of 10% SDS. Absorbance was measured using a spectrophotometer (Bio-Tek instruments, Vermont, USA) at 410nm against a standard curve of *e.coli* endotoxin (0.1-1.0EU/ml)

2.1.3 Protein precipitation from cell culture media

- 100% Acetone (Fisher Scientific, UK)
- 1% Sodium dodecyl sulphate (SDS) (Sigma Aldrich, Dorset, UK).
- Protease Inhibitor Cocktail: Serine, Cysteine and Acidic protease inhibitors (Roche Pharmaceuticals, UK).

Cell culture supernatants were harvested and proteins were precipitated using an acetone-based protocol. The principle of this technique is that the solubility of a protein in media is dependent on the dielectric constant of that solution. Typically solutions such as H_2O have a high dielectric constant caused by repulsive forces between proteins in solution to be of a threshold high enough to allow dissolution into the media. Solutions such as acetone have a significantly lower dielectric constant, and thus when combined with an H_2O based media are capable of lowering the net dielectric constant, allowing molecules to exist closer together (reduction of repulsive forces) and thus precipitating proteins.

Supernatants were combined with 5 times volume of 100% acetone, vortexed vigorously and incubated at -20°C for 3 hours. Samples were centrifuged at 14,000g for 15mins at room temperature, the resultant supernatants were aspirated and discarded, the cell pellet was then allowed to air dry at room temperature for 30mins. Protein pellets were then re-suspended in 100µl of 1% SDS containing a 10% protease inhibitor cocktail and sonicated. The protein content of the sample was quantified by BCA prior to western blot analysis as described in Section 2.1.5.

2.1.4 LIVE/DEAD viability staining

Cell viability was analysed using a commercial LIVE/DEAD kit (Invitrogen, Paisley, UK).

- 4mM Calcein AM ester in DMSO (Invitrogen, Paisley, UK)
- 2mM Ethidium Homodimer-1 in DMSO (Invitrogen, Paisley, UK)
- Dulbecco's Modified Eagles Medium DMEM (Sigma Aldrich, Dorset, UK).

Calcein-AM localises to the cytosol and is cleaved by esterases to calcein. Calcein is retained within the cytosol of viable cells and produces a green fluorescence. Ethidium intercalates between nucleotide bases in non-viable/necrotic cells and produces an intense red fluorescence.

C2C12 myotubes were cultured in 35mm dishes (Costar, UK) as previously described in Section 2.1.1. Control C2C12 myotubes or myotubes treated with TNF-α were exposed to a cocktail of 4mM calcein-AM ester and 2mM Ethidium Homodimer-1 in DMEM for 30mins at room temperature and protected from light. Cells were washed briefly in serum-free DMEM and visualised by confocal laser microscopy. Calcein was visualised using a fluoroscein optical filter, with excitation at 480 ± 10nm and ethdium visualised with a rhodamine filter at 530 ± 12.5nm. Visualisation of the two fluorophores allowed assessment of cellular viability.

2.1.5 Trypan blue exclusion assay

- Trypan blue solution (0.4%) in DMEM (Sigma Aldrich, Dorset, UK).
- 70% Ethanol (Fisher Scientific, UK).

Trypan blue is a classical means of assessing cell viability. The blue stain is taken up by non-viable cells resulting in a blue colour when viewed using light microscopy. Cell counts were carried out blind, with culture dishes divided into quartiles and 50 cells counted per region.

C2C12 myotubes in culture were combined with an equal volume of trypan blue solution. All cell viability counts were carried out randomised and blinded where possible. In addition, a sample of cells was treated with 70% ethanol for 30mins prior to treatment with trypan blue to provide a positive control.

2.1.6 Heat treatment of C2C12 myotubes

- Dulbecco's Modified Eagles Medium DMEM (Sigma Aldrich, Dorset, UK).

C2C12 myotubes were cultured into six well tissue culture plates (Costar, UK). Myotubes were raised to a temperature of 42°C in a bench-top waterbath for 1 hour in serum-free DMEM. C2C12 myotubes were harvested and cell culture media collected 24 hours later (Maglara et al., 2003). Cell culture media was subject to acetone precipitation as described in Section 2.1.3. Total protein was quantified in both the cell lysate and culture media using the BCA assay as described in Section 2.3.1. Samples were subject to SDS-PAGE and western blotting as described in Section 2.3-2.3.3.

2.2 Sample preparation

2.2.1 Exosome Isolation

- 1 X Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma Aldrich, Dorset, UK).
- 1% Sodium dodecyl sulphate (SDS) (Sigma Aldrich, Dorset, UK).
- Protease Inhibitor Cocktail (Roche Pharmaceuticals, UK).

Exosomes were isolated from C2C12 cell culture supernatants. To isolate these small, low-density vesicles it is necessary to use a differential centrifugation protocol (Valadi et al., 2007, Eldh et al., 2010).

C2C12 myotubes were cultured and differentiated to form myotubes as previously described in Section 2.1.1. Prior to culture, fetal calf serum (FCS) and horse serum (HS) used in the culture media was depleted of exosomes by ultracentrifugation at 100,000g for 90 minutes (Thery et al., 2006).

C2C12 cell culture media was removed and centrifuged at 12,000g for 20mins to remove large debris and particles. Cell culture media was filtered through a 200μm pore and centrifuged at 100,000g for 90mins using a SW40 ultracentrifuge rotor (Beckman Coulter, UK). The supernatant was then aspirated and discarded and exosome pellets were re-suspended in 100μl 1% SDS containing a protease inhibitor cocktail and stored at -80°C.

2.2.2 Isolation of the nuclear fraction of C2C12 myotubes for analysis of protein content by western blotting

- NE-PER® Nuclear and Cytoplasmic extraction reagents (Thermo Fisher Scientific, MA, USA). The kit comprised of:
- Cytoplasmic extraction reagent I (CER I).
- Cytoplasmic extraction reagent II (CER II).
- Nuclear extraction reagent I (NER).
- Additional reagents:
- Protease Inhibitor Cocktail: Serine, Cysteine and Acidic protease inhibitors (Roche Pharmaceuticals, UK).
- Dulbecco's Modified Eagles Medium DMEM (Sigma Aldrich, Dorset, UK).

C2C12 myotubes were removed using a cell scraper from 6-well tissue culture plates (Costar, UK) into 1ml of ice-cold DPBS, and centrifuged at 14,000 g for 10mins at 4°C. The cell pellet was resuspended into 100µl of CER I, vortexed vigourously for 15 seconds then incubated on ice for 30 mins. After which, 5.5µl of ice-cold CER II was added to the sample and vortexed vigourously for 15 seconds then incubated on ice for 1 mins. Sample was then centrifuged at 14,000 g for 5mins at 4°C. The supernatant was aspirated; this was saved on ice, as the cytoplasmic fraction. The remaining pellet was resuspsend into 100µl of ice-cold NER. The sample was vortexed vigorously for 15 seconds then incubated on ice for 10mins. This was repeated over a period of 40mins. The sample was then

centrifuged at 14,000 g for 10mins at 4°C. The supernatant was aspirated and retained as the nuclear fraction. Samples were stored at -80°C until analysis by western blotting.

2.2.3 Extraction of RNA from C2C12 cells

- TriReagent (Sigma Aldrich, Dorset, UK).
- Isopropanol (Sigma Aldrich, Dorset, UK).
- Chloroform (Sigma Aldrich, Dorset, UK).
- Ethanol (75%)
- Dulbecco's Phosphate buffered saline (DPBS) (Sigma Aldrich, Dorset, UK).

C2C12 myotubes were cultured *in vitro* as described in Section 2.1.1. C2C12 myotubes were harvested using a cell scraper in ice-cold DPBS and centrifuged at 14,000g. The cell pellet was re-suspended into TriReagent and allowed to stand at room temperature for 5mins. Two hundred microlitres of chloroform was added to each sample and agitated at room temperature for 15 seconds then allowed to stand for a further 15mins. Following incubation samples were centrifuged at 12,000g for 15mins at 4°C to separate the lysate into several phases: The cell lysate was separated into 3 phases, a lower (pink) organic protein containing phase, a DNA containing interphase and an upper (colourless) RNA containing aqueous phase.

The upper RNA phase was aspirated into a new tube from each sample and combined with 500µl of isopropanol. Samples were vortexed and incubated at room temperature for 10mins then centrifuged at 12,000g for 10mins at 4°C. The supernatant was aspirated and discarded for each sample and the RNA pellet washed with 75% ethanol and centrifuged at 12,000g for 5mins at 4°C. Supernatants were aspirated and discarded, and pellets air dried at room temperature for 5mins. Finally,

the RNA pellet was re-suspended into DNase/RNase free ddH_2O, re-suspension was obtained by repeated pipetting and a brief heating step to 55°C for 15mins. Samples were then stored at -80°C.

2.3 Gel electrophoresis, western blotting, ELISA. Luminex techniques and Creatine kinase assay for protein analysis

2.3.1 Bicinchoninic acid (BCA) assay

The BCA assay (Sigma Aldrich, Dorset, UK) was used to determine total protein content of samples. The kit contains:

- Reagent A: Bicinchoninic acid (BCA) solution comprised of 160mM $NaCO_3.H_2O$, 25mM BCA-Na, 7mM Na_2 tartrate & 0.95% $NaHCO_3$.
- Reagent B: 160mM $CuSO_4.5H_2O$
- Bovine serum albumin (BSA) 1mg/ml as a protein standard
- Reagent C prepared by adding 12.5ml of Reagent A to 250µl of Reagent B.

The principle of the BCA assay is based on Cu^{2+} - protein complex formation. The reduction of Cu^{2+} to Cu^{1+} is proportional to protein content of the sample. The BCA reagent then forms a purple coloured complex with Cu^{1+} which can be quantified using a spectrophotometer (Bio-Tek instruments, Vermont, USA).

Samples were analysed of standards 25-250µg/ml made from the 1mg/ml stock solution of bovine serum albumin (BSA). Twenty microlitres of samples and standards were added to a 96-well microplate. Two hundred microlitres of Reagent C was added to each well, mixed, and the microplate incubated at 50°C for 30 minutes. The absorbance of both the samples and standards was then determined using a

microplate spectrophotometer (Bio-Tek instruments, Vermont, USA) at 570nm and the protein content calculated using the standard curve.

2.3.2 Gel electrophoresis

2.3.2.1 Gel preparation

- Stock acrylamide stock solution: 30% Acrylamide, 0.8% bisacrylamide cross-link in ddH_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: 40ml stock acrylamide solution, 26ml gel buffer, and 34ml ddH_2O.
- 4% Acrylamide Solution: 13ml stock acrylamide solution, 25ml stacking buffer, 61ml ddH_2O.
- 10% (w/v) Ammonium Persulphate (APS), (Sigma Aldrich, Dorset, UK).
- NNN'N'-tetramethylethylene-diamine (TEMED), (Sigma Aldrich, Dorset, UK).

A 12% gel was prepared as detailed above, the polymerisation of the gel required 10ml of 12% acrylamide solution combined with 100µl of 10% APS and 10µl TEMED to catalyse the reaction. The gel was cast between 2 x (8x10cm) glass plates, separated by a 2mm spacer. The 12% gel was then overlaid with a 4%

stacking gel, whereby, 10ml of 4% acrylamide solution was combined with 100ml 10% APS and 25ml TEMED. A 1-1.5cm stacking gel was poured over the 12% gel and a comb positioned in place to create wells for protein loading.

2.3.2.2 Electrophoresis of proteins

- Electrophoresis (Running) Buffer: 10X Tris/Glycine/SDS (0.025M Tris, 0.192M Glycine & 0.1% (w/v) SDS; National Diagnostics, Georgia, USA).
- Lamaelli loading buffer: 46.03mg/ml SDS, 20.9% Glycerol, 2.1% (v/v) β-mercaptoethanol, 0.052mg/ml bromophenol blue in 0.128M Tris/HCl buffer, pH 6.8.

Fifty micrograms of protein was combined with an equal volume of Lamaelli loading buffer (National Diagnostics, Georgia, and USA) and boiled for 5mins. Samples were allowed to cool for 15mins and then applied to a 12% polyacrylamide gel via a 4% stacking gel. Proteins were separated by electrophoresis at a constant current of 20mA per gel using an electrophoresis tank with 1X running buffer. The electrophoresis tank was cooled throughout with a tap H_2O system.

2.3.3 Western blotting of proteins

- Anode 1 Buffer: 0.3M Tris in 20% Methanol pH 10.4.
- Anode 2 Buffer: 25mM Tris in 20% Methanol pH 10.4.
- Cathode Buffer: 40mM 6-amino Hexanoic Acid, in 20% Methanol pH 7.6.
- Ponceau S Solution: 0.1% Ponceau S (w/v) in 5% (v/v) Acetic Acid.

Once separated, proteins were transferred to a nitrocellulose membrane by a Multiphore Continuous Blotting System (Pharmacia, Uppsala, Sweden). The system comprises graphite electrodes which facilitate the current to transfer separated proteins onto a nitrocellulose membrane. The gel was placed upon a nitrocellulose membrane, sandwiched in between layers of Whatmann No. 1 filter paper which had been pre-soaked in Anode 1, 2 & Cathode buffers. A constant current over 0.8 mA cm^{-2} was applied to each gel over a 90min period (Kayani et al., 2008). Nitrocellulose membranes were treated with Ponceau S stain to confirm homogenous protein transfer.

Figure 2.2 Arrangement of the gel and nitrocellulose membrane between the two electrodes for efficient protein transfer.

2.3.3.1 Analysis of the nitrocellulose membrane for specific proteins

- PBS solution pH 7.2: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in ddH_2O, pH 7.2.
- PBS solution pH 6.0: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in ddH_2O, pH 6.0.

- PBS/Tween 20 Solution: 0.05% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20) in PBS solution.
- Blocking Solution: 5% (w/v) powdered milk in PBS/Tween.
- Ponceau S stain (Sigma Aldrich, Dorset, UK).
- SuperSignal West Dura Chemiluminesence Kit (Pierce, Illinois, USA).

To ensure equal loading of gels and successful transfer membranes were stained using Ponceau S stain. Membranes were immersed overnight in a 5% milk solution at 4°C and subsequently analysed for the presence of HSC70, HSP70, HSP60, and HSP10 using antibodies raised against these proteins (Enzo Life Sciences, Exeter, UK). Each membrane was incubated at room temperature under agitation with a 10ml antibody solution at concentrations detailed in Section 2.2 for 1 hour. Following this, each membrane underwent 3 x 10min washes with PBS/Tween pH 7.2. The membrane was then incubated for a further hour under agitation with the species specific peroxidase conjugated secondary antibody, relevant to the primary antibody of interest (Maglara et al., 2003). Each membrane was then washed for 3 x 10mins in PBS/Tween and then incubated under agitation with 10ml PBS Tween pH 6.0, for optimal chemiluminesence detection. The membrane was finally exposed to a chemiluminesence agent using a SuperSignal West Dura Kit (Pierce, Illinois, USA). The nitrocellulose membrane was placed between two sheets of acetate and analysed using a Bio-Rad Chemi-doc XRS system with QuantityOne software (Bio-Rad, Hercules, USA). The intensity of protein bands was quantified using densitometry; exposure was carried out ensuring linear intensity and to prevent pixel saturation.

2.3.3.2 Antibodies

Antibody	Company	Catalogue No.	Species	Dilution
HSP10	Enzo Life Sciences	Ab53106	Rabbit (Polyclonal)	1:1000
HSP60	Enzo Life Sciences	SPA-807	Mouse (Monoclonal)	1:5000
HSP70	Enzo Life Sciences	SPA-810	Mouse (Monoclonal)	1:1000
HSC70	Enzo Life Sciences	SPA-815	Rat (Monoclonal)	1:1000
CD9	Abcam	Ab19761	Mouse (Monoclonal)	1:1000
p65 NFkB	Abcam	Ab31481	Rabbit (Polyclonal)	1:2000

Species specific secondary antibodies used were conjugated to horseradish peroxidase to allow detection using a chemiluminesence agent (Pierce, Illinois, USA).

2.3.3.3 Antibody removal and membrane re-probing

- PBS/Tween solution, pH 7.2
- 62.5mM Tris/HCl, pH 6.7
- 2% (w/v) SDS (Sigma Aldrich, Dorset, UK)
- 100mM β-mercaptoethanol (Sigma Aldrich, Dorset, UK)

For analyses of additional proteins antibodies bound to the nitrocellulose membrane were removed following chemilumiscent exposure. Nitrocellulose membranes were incubated in a solution of PBS/Tween containing 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris/HCl, pH 6.7 at 50°C for 30mins. The membrane was then washed for 3x10min in PBS/Tween pH 7.2 at room temperature, following which the nitrocellulose was re-blocked with 5% milk solution and protein analysis continued as described in Section 2.1.5.4.

2.3.4 HSP analysis by ELISA

- Murine Recombinant Heat Shock Protein 70 (Enzo Life Sciences, Exeter, UK).
- HSP70 ELISA kit (Cambridge Bioscience, UK).
- HSP60 ELISA kit (Enzo Life Sciences, Exeter, UK).
- Dulbecco's Phosphate buffered saline (DPBS, Sigma Aldrich, Dorset, UK).

Conditioned media was obtained using the cell culture protocol as previously described in Section 2.1.1; HSP70 was added at a concentration of 13ng/ml to 6-well tissue culture plates (Costar, UK) and incubated at 37°C for 3, 8 or 18hrs. Levels of HSP70 added into the conditioned media were consistent with those detected to be release from cultured cells and detected *in vivo*. 100µl of supernatant was assayed using a High-Sensitivity HSP70 ELISA kit (Cambridge Bioscience, UK) and analysed according to the manufacturers' protocol.

2.3.5 Bioplex multi-bead analysis

Reagents:

- Assay Buffer (PBS- 0.05% Tween20 pH 7.4, 0.1% BSA, 0.01% NaN_3).
- Wash Buffer (PBS- 0.05% Tween20 pH 7.4).
- Streptavidin-phycoerythrin fluorophore (Bio-Rad, Heracules, USA).
- 96-well nano-pore filter membrane microplate (Millipore, Massachusetts, USA).
- Bioplex single plex (mouse) cytokine magnetic beads and detection antibody (Bio-Rad, Heracules, USA)

Cytokines were measured in cell culture media, cell lysates, muscle homogenates, murine serum and microdialysates using a multiplex bead analysis technique (BioRad, Heracules, USA). A 96-well filter plate was initially pre-wetted with 200µl of assay buffer, following which the liquid was vacuum aspirated. Samples and standards which comprised a cocktail of lyophilised protein of known mouse cytokines were incubated with sets of magnetic spheres (each with a different target antibody attached) on a filter membrane 96-well plate for 30mins under constant agitation. Samples and standards were then washed 3 times in a PBS-tween solution pH 7.2 wash buffer using a vacuum filtration technique calibrated to a suction pressure of 1-3mmHg. Briefly, 200µl of wash buffer was added to each well, the plate was then placed onto the vacuum filtration platform. Pressure was applied to the plate to ensure a tight seal and the supernatant aspirated.

A complementary detection antibody was then incubated with the samples and standards under agitation (300rpm) for a further 30mins. Following which 3 further wash steps were performed under vacuum filtration. Finally, samples and standards

were incubated for with a streptavidin-phycoerythrin fluorophore (protected from light) for 10mins under agitation (300rpm). The samples and standards were then washed once again and analysed using the Bioplex 200 platform (Bio-Rad, Heracules, USA).

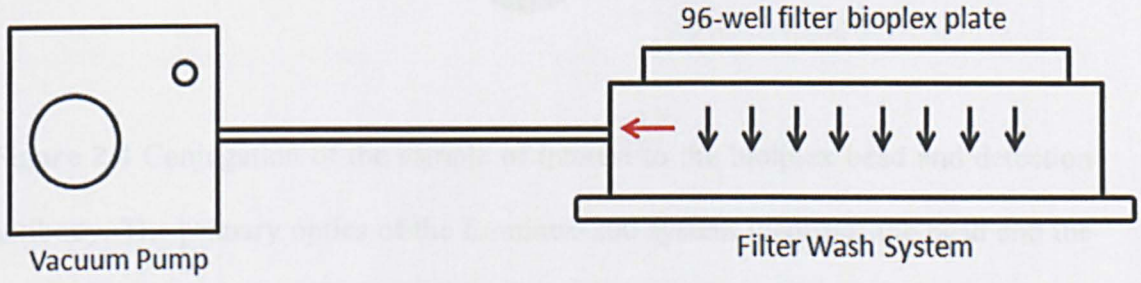


Figure 2.3 Luminex plate washing system: A vacuum pressure of 1-3mmHg applied to the plate allows aspiration of the supernatant from each well of the filter plate and retention of the bead-sample complex on the plate membrane.

The Bioplex 200 apparatus uses flow-cytometry based technology. A 50µl aliquot of samples/magnetic beads are aspirated out of each individual well, the primary optics then identifies each bead according to the colour (bead-region) assigned for each individual cytokine. The secondary optics analyse the presence and intensity of the Strep-PE fluorophore, quantified as mean fluoresence intensity (MFI), comparing this to the MFI of the known protein standard (Figure 2.4) for each target allows quantification of multi cytokines in each individual sample using Bioplex 5 software (Bio-Rad, Heracules, USA) .

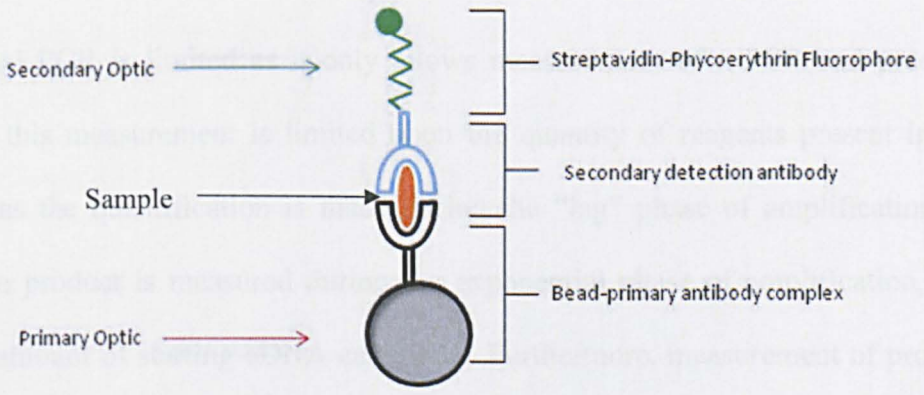


Figure 2.4 Conjugation of the sample of interest to the biolplex bead and detection antibody. The primary optics of the Luminex-200 system identifies the bead and the antibody bound to it. The secondary optic measures the fluorescence intensity via a fluorophore bound to the detection antibody, which forms the final part of the sandwich immunoassay.

2.4 qPCR

Traditional PCR is limited as it only allows measurement of a PCR end product, however this measurement is limited upon the quantity of reagents present in the reaction as the quantification is made during the "lag" phase of amplification. In qPCR the product is measured during the exponential phase of amplification, and thus the amount of starting cDNA calculated. Furthermore, measurement of product during the exponential phase of the reaction is favourable as levels of reagents are not a limiting factor.

The quantification step of qPCR is reliant on a chemical reaction during the amplification cycles using the SYBR Green I fluorescent chemistry. As the template cDNA is copied molecules of SYBR Green I are incorporated into the minor groove of the double-stranded DNA. The incorporation of the SYBR Green I into the DNA strands is typified by an increase in fluorescence, further amplicon copies produced by each PCR cycle results in amplification of the fluorescence intensity. The fluorescence intensity is analysed at the end of each PCR cycle, and is represented graphically, plotting the fluorescence intensity against the cycle number. By doing this, the starting quantity of cDNA can be calculated from the cycle at which the fluorescence intensity increases above a pre-determined "threshold" – this is termed the threshold cycle (Ct).

Once the PCR reaction is completed a melt or dissociation curve (Figure 2.5) can be produced. This analysis allows determination of the specificity of the primer annealing and amplification stages of the PCR reaction. The denaturation of the double stranded DNA occurs at varying temperatures based upon the strand length and its guanine/cytosine composition. It is possible to analyse several different

amplicon products produced in one reaction. The several products may be due to splice variants in the gene being analysed, unspecific primer annealing and primer dimerisation. However, quantification and analysis can only occur if only one product is amplified.

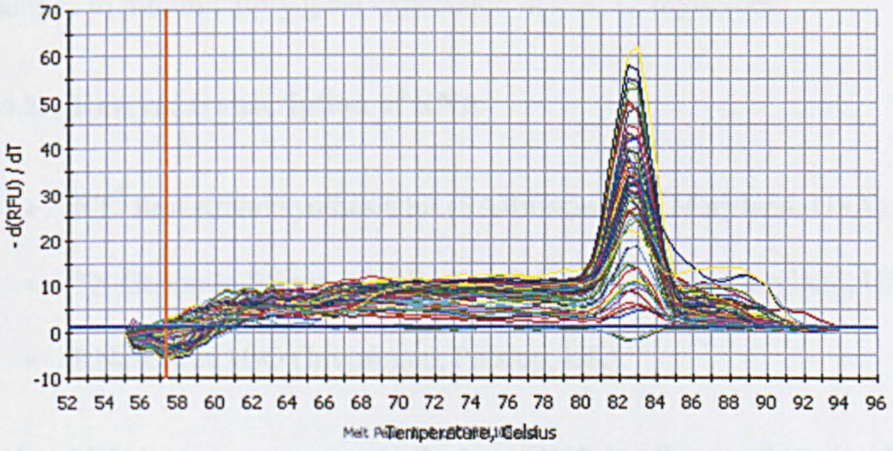


Figure 2.5 Example of a qPCR melt curve showing amplification of individual genes of interest, giving an indication of primer affinity and the overall denaturing and annealing process.

2.4.1 qPCR array analysis of RNA to determine changes in inflammatory gene expression in control C2C12 myotubes and myotubes following TNF-α treatment.

Quantitative Polymerase Chain Reaction arrays (qPCR) were used to analyse changes in inflammatory gene expression in C2C12 myotubes.

2.4.1.1 Reverse transcription of RNA

- RT^2 first strand synthesis kit (SABiosciences, Maryland, USA).
- 5X Genomic Elimination Buffer (SABiosciences, Maryland, USA).
- RNase-free H_2O (Invitrogen, Paisley, UK).

Isolated RNA was reverse transcribed to cDNA to allow analysis in qPCR reactions. The transcription process was carried out using RT^2 first strand kit. Firstly contaminating genomic DNA was removed by combination of 2mg of template RNA with 5x genomic DNA elimination buffer with RNase free H_2O up to a total volume of 10μl. To ensure successful depletion of gDNA samples were analysed using a spectrophotometer at 260/280nm for the presence of gDNA (Eppendorf, Biophotometer, USA). The reaction mixture was prepared and heated to 42°C for 5mins and immediately cooled on ice for 1 minute. A reverse transcription reaction mixture was prepared as follows for each 10μl gDNA elimination mixture:

- 4μl 5X RT Buffer 3
- 1μl Primer & External Control Mix
- 2μl RT Enzyme mix 3
- 3μl RNase-free H_2O

Using a PCR thermocycler (Bio-Rad, iCycler, Bio-Rad, Hercules) the reaction mixture was then heated to 42°C for 15mins, following which, heating to 95°C for 5mins degraded the RNA and activated the reverse transcriptase enzyme. The reaction mixtures were then stored at -20°C.

2.4.1.2 Primers

- Murine chemokines and receptors array (SABiosciences, Maryland, USA)

A commercial qPCR array kit was used to analyse changes in gene expression of a large set of inflammatory cytokines and receptors. Ninety-six genes in total were analysed, together with 5 housekeeping genes. Primers for all target genes were bound to a 96-well PCR array plate.

- Chemokine genes:

  Ccl1, Ccl11, Ccl12, Ccl17, Ccl19, Ccl2, Ccl20, Ccl22, Ccl24, Ccl25, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl8, Ccl9, Cx3cl1, Cxcl1, Cxcl10, Cxcl11, Cxcl12 (Sdf1), Cxcl13, Cxcl15, Cxcl4, Cxcl5, Cxcl9, Il13.

- Chemokine receptors:

  Ccr1, Ccr2, Ccr3, Ccr4, Ccr5, Ccr6, Ccr7, Ccr8, Ccr9, Cxcr3, Il8rb, Xcr1.

- Cytokine genes:

  Ifng (IFNγ), Il10, Il11, Il13, Il15, Il16, Il17b, Il18, Il1a, Il1b, Il1f6, Il1f8, Il20, Il3, Il4, Itgam, Itgb2, Lta, Ltb, Mif, Scye1, Spp1, Tgfb1, Tnf, Cd40lg.

- Cytokine receptors:

  Ifng (IFNγ), Il10ra, Il10rb, Il13, Il13ra1, Il1r1, Il1r2, Il2rb, Il2rg, Il5ra, Il6ra, Il6st, Tnfrsf1a (TNFR1), Tnfrsf1b (TNFR2).

- Other genes involved in inflammatory response:

Abcf1, Bcl6, Blr1, C3, Casp1, Crp, Il1r1, Il8rb, Tollip.

- Housekeeping genes:

  Gusb, Hprt1, HSP90ab1, Gapdh, Beta-actin.

2.4.1.3 qPCR reactions

- Superarrays RT^2 qPCR Master Mix
- RNase-free dH_2O
- cDNA

The PCR reaction was carried out using a Bio-Rad thermocycler. cDNA synthesised using the method described in Section 2.2.1.5 was combined in the following mixture:

- 20µl of 0.5µg cDNA
- 1305µl RNase-free dH_2O
- 1375µl of RT^2 qPCR Master Mix (SABiosciences, USA).

Twenty five microlitres of reaction mixture was added to each well of the 96-wells of the array plate. The plate was then securely sealed and subject to the following experimental cycles in a thermocycler (Figure 2.6):

- 1 cycle for 10mins at 95°C
- 40 cycles. Each cycle comprises 15 seconds at 95°C followed by 30 to 40 sec at 55°C and 30 sec at 72°C

The fluorescence from the SYBR green incorporation was quantified throughout each cycle to obtain the threshold cycle (Ct) for each gene. Quantification of changes in gene expression was carried out using the delta-delta ct (ΔΔct) method. This method calculates changes in gene expression between the control and experimental samples in both the house keeping genes and genes of interest, expressing data as fold change.

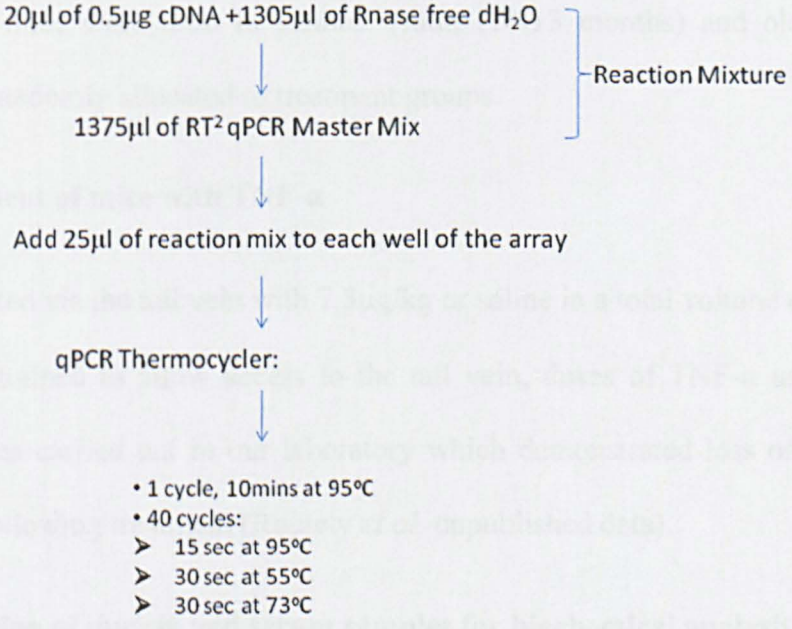


Figure 2.6 Schematic representations of the qPCR array preparation and reaction.

2.5 Mice and protocols

2.5.1 Mice

Mice were fed on a standard laboratory diet (Standard Rat and Mouse Breeder and Grower Pelleted diet, CRM (P), Special Diet Service, UK) and subjected to a 12 h light: 12 h dark cycle. Experiments were performed in accordance with UK Home Office Guidelines under the UK Animals (Scientific Procedures) Act 1986. Wildtype (WT) C57Bl6 mice were used in studies. Adult (10-13 months) and old (22-24 months) were randomly allocated to treatment groups.

2.5.1.1 Treatment of mice with TNF-α

Mice were treated via the tail vein with 7.5μg/kg or saline in a total volume of 200μl. Mice were restrained to allow access to the tail vein, doses of TNF-α used were based on studies carried out in our laboratory which demonstrated loss of specific muscle force following treatment (Routely *et al.* unpublished data).

2.5.2 Preparation of muscle and serum samples for biochemical analysis

- 1% Sodium dodecyl sulphate (SDS) (Sigma Aldrich, Dorset, UK).
- Protease Inhibitor Cocktail: Serine, Cysteine and Acidic protease inhibitors (Roche Pharmaceuticals, UK).

The *gastrocnemius* muscle was removed from the hindlimb of adult and old mice treated with TNF or saline and ground under liquid nitrogen. Muscle powder was resuspend in 1% SDS with a protease inhibitors and subject to mechanical homogenization and centrifugation 14,000g. Samples were analysed for HSP content

by western blotting as described in Section 2.1.9. Blood was taken from the inferior vena cava and allowed to clot a room temperature for 1 hour. Samples were centrifuged at 12,000g for 15mins. Serum was aspirated and stored at -80°C. Serum taken from TNF-α treated mice were analysed for the presence of both HSP60 and HSP70, by ELISA according to the manufacturers' protocol.

2.5.3 Isolation and treatment of muscle fibres from *flexor digitorum brevis* (FDB) muscle

- Type H Collagenase- (Sigma Aldrich, Dorset, UK)
- Minimum Essential Media MEM (Sigma Aldrich, Dorset, UK)
- Fetal Calf Serum FCS (Invitrogen, UK)

Six C57Bl6 WT mice aged 10-12 months and 22-24 months were randomly allocated into treatment and placebo groups. Mice were killed by cervical dislocation and the *Flexor Digitorum Brevis* (FDB) muscle of each hind limb exposed and removed. The FDB was then placed in a solution of Collagenase H solution (0.4%) in MEM containing 10% FCS, 2mM L-glutamine, and 50i.u penicillin and 50μg/ml streptomycin. Incubation in Collagenase H solution was carried out over 2 hours at 37°C with periodical mechanical agitation at 30 minute to help dissociation of fibres and digestion of collagen. Any undigested fibre bundles were triturated using a wide-bore pipette (Sakellariou et al., 2011). Fibres were then centrifuged at 600g for 30 seconds to remove debris. Fibres were then plated onto 35mm dishes in serum free MEM and allowed to settle for 1 hour. Fibres were then treated with TNF-α (25ng/ml) in 2ml MEM for 3 hours. Media and fibres were harvested and stored at -70°C.

Cell culture media from isolated fibres treated with TNF-α were analysed for the presence of both HSP60 and HSP70, according to the manufacturers' protocol as described in Section 2.3.4.

2.6 Statistics

For comparison of two variables a student's t-test was performed. For analysis of multiple variables One-way ANOVA with tukey post-hoc was used. PASW 17 software (formerly SPSS) was used to perform all analyses. A P-value <0.05 was considered to be significant, data are expressed ± SEM.

CHAPTER 3

THE EFFECT OF TNF-α TREATMENT ON THE HEAT SHOCK RESPONSE IN C2C12 MYOTUBES; MUSCLE AS A SOURCE OF EXTRACELLULAR HSPS?

3.1 INTRODUCTION

3.1.1 A key component of systemic inflammation during sepsis is elevated serum TNF-α.

Sepsis is a hyper-inflammatory state characterised by the exposure of cells, tissues and organs of the body to a barrage of pro-inflammatory cytokines. This effect is commonly initiated in response to profound gram-negative bacterial infection. A key component of this hyper-inflammatory state is Tumour Necrosis Factor-alpha (TNF-α). TNF-α was originally termed cachectin due to its well defined role as a key pro-inflammatory cytokine in cachexia (Beutler et al., 1985).

3.1.2 Elevated TNF-α plays a major role in muscle atrophy and dysfunction.

The catabolic effect of TNF-α on muscle has been well characterised. Treatment of muscle cells with TNF-α induces loss of total protein (Li et al., 1998) and up-regulation of ubiquitin E3 ligases such as atrogin1/MAFbx involved in protein degradation (Li et al., 2005, Moylan et al., 2008). More recently, an association has been demonstrated between loss of cardiac muscle mass and function and elevated circulating levels of cytokines (Toth et al., 2006).

Disruption of the ubiquitin/proteasome pathway has been widely described as a pivotal part of this muscle wasting (Mitch and Goldberg, 1996). Additional research has indicated TNF-α to have a positive role in muscle, promoting protein synthesis in C2C12 myotubes (Plaisance et al., 2008). In addition to the catabolic/anabolic function, TNF-α induces up-regulation in gene expression of several inflammatory cytokines and receptors in C2C12 myotubes (Alvarez et al., 2002a).

The overall catabolic effect of TNF-α has a profound clinical impact, particularly with respect to severely ill septic patients. Typically, a sedentary septic patient loses 0.5-1% body mass per day (Hasselgren et al., 2005). Over a 3 week period severely septic patients can lose on average 16% total body protein, which contributes to approximately 3kg loss in body mass (Finn et al., 1996). Typically loss of muscle mass is associated with poor clinical outcome and increased mortality, particularly in terms of weaning from mechanical ventilation as a result of muscle loss and weakness (Leijten et al., 1995).

3.1.3 Elevated intracellular levels of HSPs protect muscle against inflammation.

Although TNF-α has a profound deleterious effect upon skeletal muscle, muscle has the capacity to respond and adapt to such stress. One of the many cytoprotective mechanisms evident in skeletal muscle is the expression of molecular chaperones, termed Heat Shock Proteins (HSPs). HSPs are a family of proteins which are highly conserved across species (Hartl, 1996). The primary function of HSPs is to stabilise the folding and conformational organisation of newly synthesised peptides (Fink, 1999).

Under instances of stress (including mechanical, pharmacological or bactericidal), the expression of HSPs is up-regulated and this increased cellular HSP content provides protection against further stresses (Fink, 1999, Lee et al., 2007). This cytoprotection is evident in skeletal muscle, whereby pharmacological up-regulation of HSPs using 17-(allylamino)-17-demethoxygeldanamcyin (17AAG) protects skeletal muscle against contraction induced damage (Kayani et al., 2008). Furthermore, lifelong over-expression of HSP70 in mice abrogates the age-related loss of muscle mass and function (McArdle et al., 2004). The prevention of age-

related functional loss has been further shown with over-expression of mitochondrial HSP10 in muscles of old mice (Kayani et al., 2010). The mechanism by which HSPs provide such protection are unknown but, elevation of intracellular expression of HSPs in monocytes and macrophages has been shown to suppress circulating levels of cytokines, such as IL-1β (Schmidt and Abdulla, 1988) and TNF-α (Ribeiro et al., 1996).

Further indirect evidence for a role of HSPs in providing protection against inflammation comes from work using glutamine. Supplementation of rats with glutamine up-regulates HSP expression in skeletal muscle (Wischmeyer et al., 2001) and glutamine supplementation reduced mortality in humans following septic insult (Singleton et al., 2005). The work carried out by Wischmeyer and colleagues proposes that HSP up-regulation is a primary effect of glutamine. This is an area of significant interest, in terms of a therapeutic intervention via the nutritional supplementation of critically ill patients (Wischmeyer, 2006, Singleton and Wischmeyer, 2008).

Thus, the chaperone function of HSPs is one that has been widely explored in an effort to understand the possible cytoprotective abilities for treatment of a whole host of diseases and disorders (Otaka et al., 2006); (Kim et al., 2006). However over the last decade a new perspective towards HSPs has developed, whereby a population of HSPs are released into the extracellular environment and act in a hormone-like fashion has attracted significant interest (Asea, 2008a).

3.1.4 Role of extracellular HSPS in mediating systemic inflammation and muscle function.

Extracellular HSPs (eHSPs) have been shown to diverge from the chaperone function of intracellular HSPs to one of immunostimulation and immunomodulation (Asea, 2005). eHSPs have been termed "chaperokines" demonstrating both chaperone and cytokine-like properties (Asea, 2008b). It is unclear whether intracellular HSPs have an identical tertiary structure to that of eHSPs. The first major contribution to this area of research demonstrated the ability of eHSP70 to interact in a CD14-dependent manner with human monocytes inducing the release of cytokines IL-1β, IL-6 and TNF-α (Asea et al., 2000b). This cytokine-like function was initiated via Toll-like receptors (TLRs); whereby HSP60 was firstly shown to interact with B-cells via TLR-4 (Cohen-Sfady et al., 2005). Further work indicated that HSP60 augmented CD4+ and CD25+ T-cell function in a TLR-dependent manner (Zanin-Zhorov et al., 2006). Elevated circulating levels of HSP60 in the serum of septic children compared with healthy controls further suggested that (given the immunostimulatory functions of eHSPs) eHSPs may play a role in the pathogenicity of sepsis (Wheeler et al., 2007).

Little is known about the tissue source of eHSPs and the mechanisms by which HSPs are released into the extracellular environment are also poorly understood. HSPs lack a leader sequence in their peptide structure, therefore they are unable to be externalised by a classical golgi-mediated means of release. Non-specific release as a result of apoptosis and necrosis has been suggested, however, significant research in other cells has indicated that HSPs are actively released from viable cells via a non-classical pathway, termed exosomes (Gupta and Knowlton, 2007, Merendino et al., 2010).

Exosomes are small nano-sized vesicles, 50-90nm in diameter of endocytic origin, formed by inward budding of the outer membrane of late endosomes (Johnstone, 2006). Significant interest in exosome biology has developed over the past 20 years with their role as biological messengers, capable of genetic transfer between cells (Valadi et al., 2007) as well as having co-stimulatory effects on the immune system (Raposo et al., 1996). Exosomes are enriched in proteins from the cell of origin, however, numerous common proteins are found in almost all exosomes. Typically exosomes contain membrane spanning proteins termed tetraspanins, in particular CD9, CD63 and CD81 and as such these proteins are now widely described as classical exosome markers (Eldh et al., 2010).

Exosomes are released by numerous cell types: Dendritic cells (Zitvogel et al., 1998) (Thery et al., 1999), Mast cells (Raposo et al., 1997) (Valadi et al., 2007) and B- and T-cells (Clayton et al., 2005, Raposo et al., 1996) (Blanchard et al., 2002). Furthermore exosomes have been described as vehicles for HSP60 transport from cardiac myocytes in a model of myocardial injury (Gupta and Knowlton, 2007), alongside HSP70 export from endothelial cells (Zhan et al., 2009). A recent study has shown exosome release from C2C12 myoblasts during the early stages of development prior to cellular maturation (Guescini et al., 2010).

3.1.5 AIMS

The aims of the study were to:

- Develop a stable and viable model of exposure of murine C2C12 myotubes to TNF-α.
- Examine the intracellular Heat Shock Protein (HSP) content of C2C12 myotubes following treatment with TNF-α.
- Determine the ability of C2C12 myotubes to act as a source of extracellular HSPs.
- Elucidate the mechanisms of HSP release from C2C12 myotubes.
- Determine whether physical augmentation of intracellular HSPs results in modified HSP release from C2C12 myotubes following hyperthermia, thus identifying whether changes in intracellular HSPs drive eHSP levels.

3.2 METHODS

3.2.1 Establishment of the muscle cell culture model of inflammation.

Murine C2C12 myoblasts (Yaffe and Saxel, 1977) were divided in culture until they reached approximately 60-70% confluency. Cells were then differentiated over 7-days to produce mature myotubes as described in Section 2.1.1. Myotubes were treated with 10, 25 and 50ng/ml of endotoxin-free TNF-α for up to 8 hours.

3.2.2 Choice of concentration of TNF-α

The doses of TNF-α used in our studies are consistent with work in field examining the effect of TNF-α on C2C12 myoblasts and myotubes (Guttridge et al., 2000, Li and Schwartz, 2001, Frost et al., 2003, Williamson et al., 2005, Magee et al., 2008, Tang et al., 2010, Bhatnagar et al., 2010). Similar doses to those used in this study

have induced changes in protein synthesis pathways associated with cachexia, having a significant catabolic effect upon skeletal muscle (Guttridge et al., 2000, Remels et al., 2010). Although levels used *in vitro* are markedly higher than those occurring in circulation we believe the levels exposed to muscle in the vasculature and interstitial space are significantly higher than those in the blood. Thus, this suggests the doses used are representative of the levels of TNF-α existing at the muscle level in the critically ill.Furthermore, TNF-α in this range has a profound effect on myogenesis, processes which are present in and contribute to muscle dysfunction in the critically ill (Langen et al., 2001, Langen et al., 2002).

3.2.3 Assessment of muscle cell viability

Cellular viability was assessed via light-microscopy to determine any gross morphological changes. Trypan blue staining and LIVE/DEAD viability staining was carried out periodically as described in Sections 2.1.4 and 2.1.5. The presence of cleaved caspase-3 as a marker of apoptosis (Ryan et al., 2011) was examined using western blotting described in Section 2.3.3. The importance of investigating cell viability was to ensure there was no necrotic or apoptotic cell death, which would give rise to non-specific release of cellular contents into the cell culture media.

3.2.4 Stability of HSPs in culture media.

The stability of HSPs in the extracellular environment was assessed by adding physiological levels (Bathaie et al., 2010, Zhang et al., 2010b) of a recombinant HSP70 protein (13ng/ml) to conditioned media taken from C2C12 myotubes treated with 25ng/ml TNF-α for 3 hours in culture and incubated at 37°C and 5% CO_2. Conditioned media is therefore defined as culture media taken from TNF-α treated C2C12 myotubes.

Levels of HSP70 were then assessed immediately (0) and at 3, 8 and 18 hours after addition to conditioned media by ELISA as described in Section 2.1.6. The importance of determining the stability of HSPs in the cell culture media was a key component to understanding whether the observed HSP content in the media following TNF-α treatment reflected the actual amount released in response to TNF-α for that period of time.

3.2.5 Analysis of HSP content of C2C12 myotubes and cell culture media

C2C12 myotubes were treated as described in Section 2.1.2. Following TNF-α treatment the culture media was collected and subject to an acetone precipitation regime as described in Section 2.1.3. Remaining adherent myotubes were then removed into 1ml of ice-cold phosphate buffered saline (PBS), cells were then pelleted at 14,000 g at 4°C for 10mins, the supernatant discarded and the pellet resuspended in a 1% SDS solution. Total protein was quantified in both the cell lysate and precipitated cell culture media using the Bicinchoninic acid assay (BCA assay) as described in Section 2.3.1. Analysis of HSP content was carried out by SDS-PAGE and western blotting, as described in Sections 2.3-2.3.3. The nitrocellulose membrane was analysed with antibodies to the HSP family (HSP10, 60, HSC70 and HSP70) and images obtained using a chemiluminescent detection method as described in Section 2.3.3.1. Heat shock proteins were quantified by densitometry of detected bands using QuantityOne software (BioRad, Heracules, USA).

3.2.6 Determination of the mechanism of HSP release from C2C12 myotubes

C2C12 myotubes were pre-treated with Brefeldin A (BFA) a compound known to block golgi-mediated protein release (Nebenfuhr et al., 2002) or dimethyl amiloride (DMA) a known exosomal pathway inhibitor (Gupta and Knowlton, 2007) for 1 hour prior to exposure to TNF-α for 3 hours. Cell culture media was collected and subject to a several stage ultracentrifugation and filtration process to isolate exosome vesicles, as described in Section 2.2.1 (Valadi et al., 2007). Pellets from the exosomal fraction were subject to an acetone precipitation regime as described in Section 2.1.3. Finally, exosomes were re-suspended in a 1% SDS solution and total protein quantified using a BCA assay described in Section 2.3.1. Fifty micrograms of exosomal protein was separated using SDS-PAGE and subject to western blotting as described in Section 2.3. The content of HSP10, 60, HSC70 and HSP70 was determined using HSP specific antibodies and the evidence of the presence of exosomes by using an exosomal specific antibody, CD9 (Eldh et al., 2010).

3.2.7 Determination of the effect elevated intracellular HSP on the release of HSPs by C2C12 myotubes

C2C12 myotubes were heated at 42°C using a waterbath for 1 hour to increase intracellular HSP content in C2C12 cells (Maglara et al., 2003) as described in Section 2.1.6.

Whole cell lysates were collected into 1ml PBS; cell culture media was collected and subject to an acetone precipitation regime as described in Section 2.1.3 samples were subject to SDS-PAGE and western blotting, Section 2.3. The content of HSP10, 60,

C70 and HSP70 was determined using specific antibodies and and the evidence of the presence of exosomes by using an exosomal specific antibody, CD9.

3.3 RESULTS

3.3.1 Determination of cellular viability.

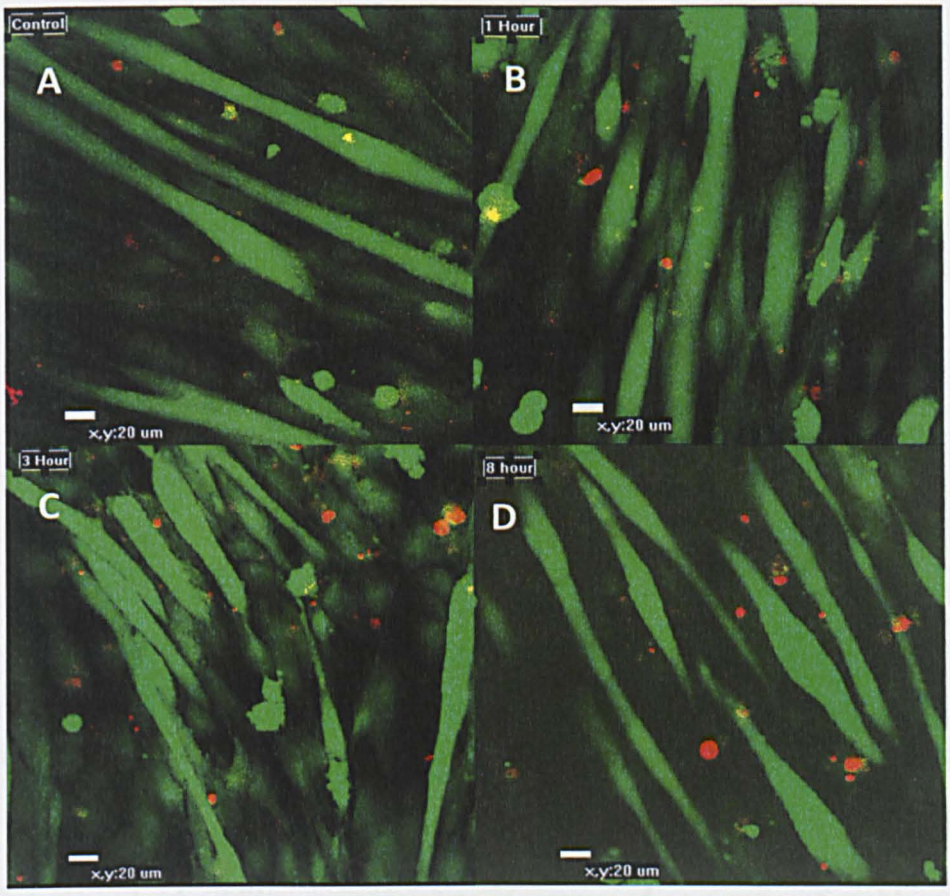


Figure 3.1 Untreated **(A)** C2C12 myotubes, and myotubes treated for 1 hour **(B)**, 3 hours **(C)** and 8 hours **(D)** with TNF-α. The green stain represents the viable myotubes which have internalised the Calcein-AM ester, the red stain is the ethidium, indicating necrosis by intercalation between DNA bases. Analyses were carried out blind, 30mm culture dishes were divided in to quartiles and fluoresence measured individually to negate any possible bias in selection. Data is a representive confocal microscopy image image for each time-point (n=6).

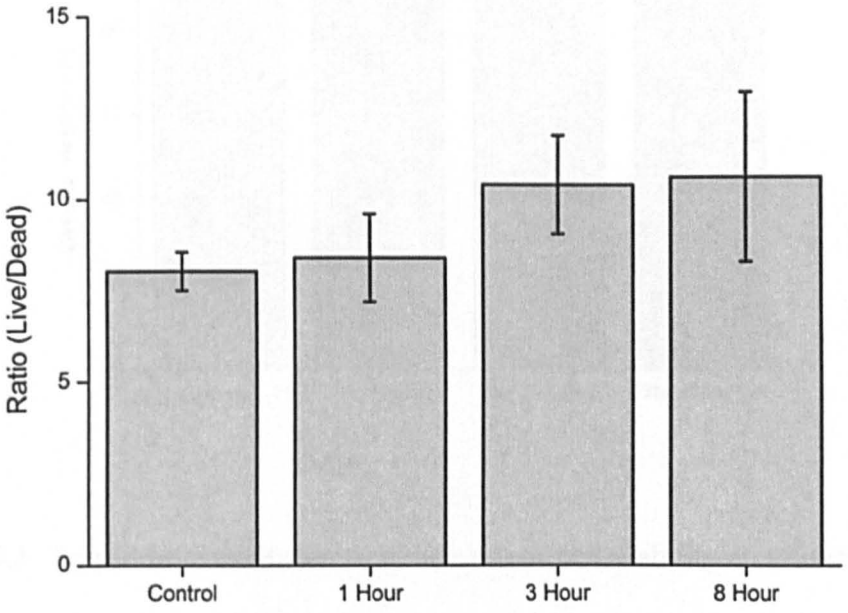


Figure 3.2 Fluoresence intensity of calcein-AM (live cells) and ethidium (necrotic cells) from C2C12 myotubes treated with TNF-α (50ng/ml) for 1, 3 or 8 hours and subject to live dead vibility analysis, data is expressed as a ratio of viable:necrotic cells. Data are represented as mean ± SEM (n=6).

Data shows no significant change in the ratio of viable to necrotic cells, in response to treatment of C2C12 myotubes with 50ng/ml of TNF-α, for up to 8 hours (Figure 3.2).

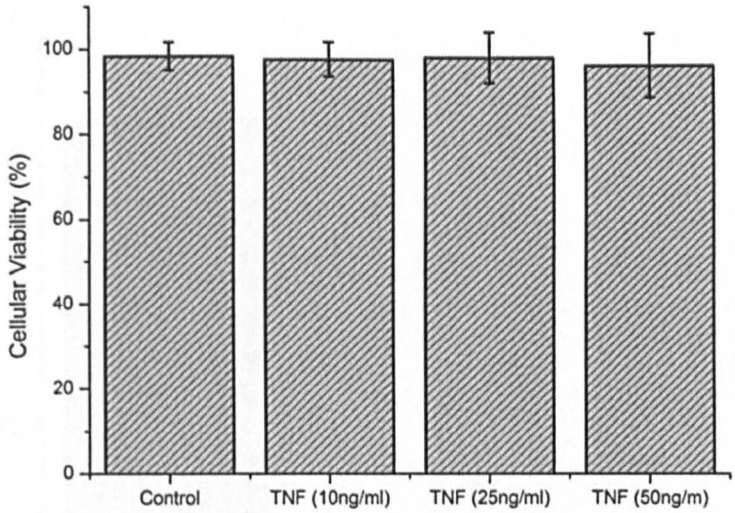


Figure 3.3 Trypan blue exclusion as a measure of cell viability at 3 hours following treatment of C2C12 myotubeswith TNF-α, data presented as mean ± SEM, (n=6).

No significant loss of cellular viability was seen in C2C12 myorubes in response to concentrations of TNF-α up to 50ng/ml when using LIVE/DEAD assay (Figure 3.1) and trypan blue exclusion methods (Figure 3.3).

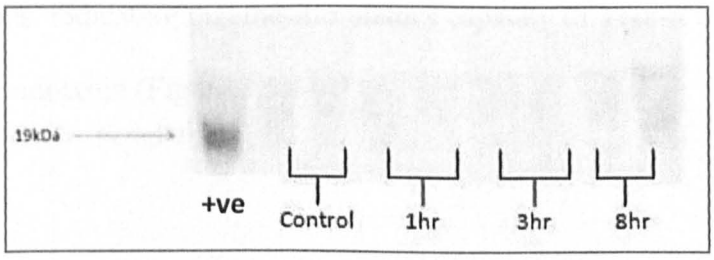


Figure 3.4 Representative western blot of cleaved caspase-3, 1, 3 and 8 hours following treatment of C2C12 myotubes with TNF-α (25ng/ml).

No detectable levels of cleaved caspase-3 (Figure 3.4) was observed in cell lysate from C2C12 following treatment with TNF-α.

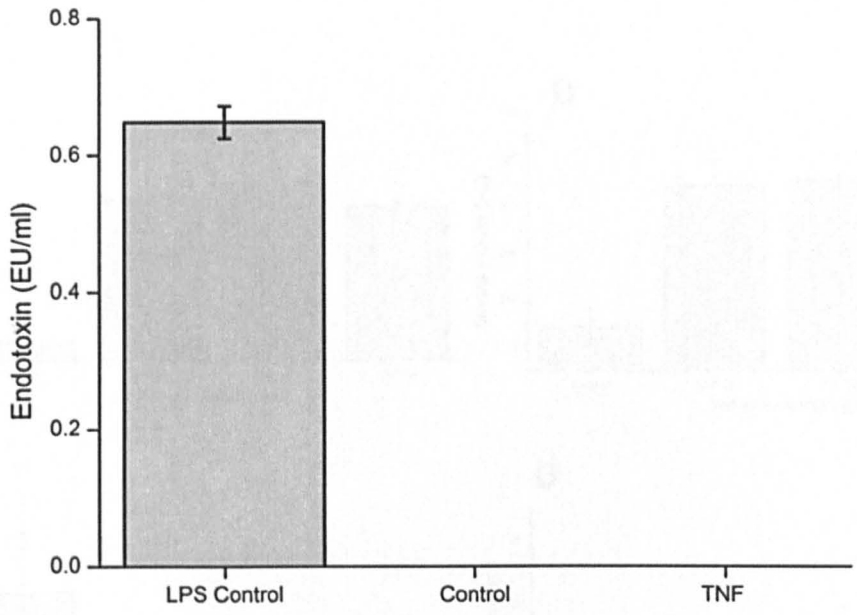


Figure 3.5 Endotoxin levels present in culture media using preparation of LPS (1μg/ml) as a positive control, and TNF (25ng/ml). Endotoxin levels were derived from a known *e.coli* based standard. Data are presented as mean ± SEM (n=6) (*p≤0.05).

No detectable levels of endotoxin were present in the control media or TNF-α preparation. Thus, indicating that the stimulatory capacity of TNF-α is not due to any contaminating endotxoin (Figure 3.5).

3.3.2 The effect of TNF-α treatment on HSP content of C2C12 myotubes

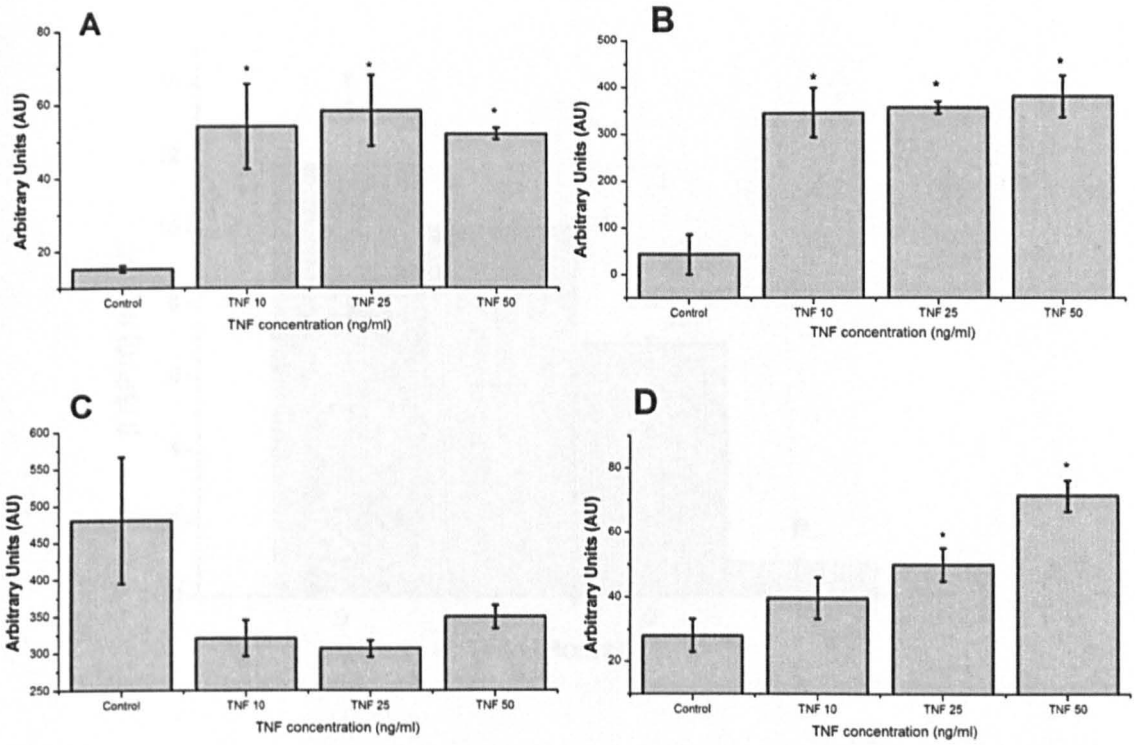


Figure 3.6 HSP60 (**A**), HSP10 (**B**), HSC70 (**C**) and HSP70 (**D**) of control untreated C2C12 myotubes and of myotubes following 3 hours of treatment with either 10, 25 or 50ng/ml of TNF-α. Data are presented as mean ± SEM (n=5); *p ≤ 0.05 compared with control cells.

Data demonstrate significant elevation in HSP10, HSP60 and HSP70 content of C2C12 myotubes when treated with 25ng/ml TNF-α, but no significant change in HSC70 was seen at any concentration of TNF-α (Figure 3.6).

3.3.3 The stability of HSPs in cell culture media

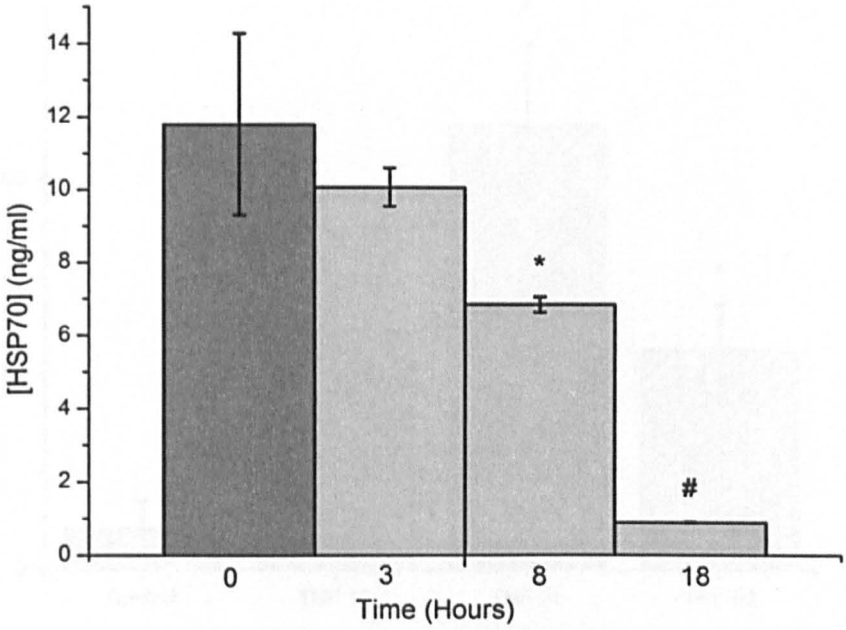


Figure 3.7 Concentration of recombinant HSP70 after incubation in conditioned media at 37°C and 5% CO_2 for 3, 8 and 18 hours from a starting concentration of 13ng/ml. Data are presented as mean ± SEM (n=6) (*$p \leq 0.05$; #$p \leq 0.01$ compared with immediately following adition to the conditioned media

No significant reduction in HSP70 concentration was observed following 3 hours of incubation of HSP70 in conditioned media at 37°C and 5% CO_2. In contrast, significant reductions in HSP70 concentrations were seen following incubation in conditioned media for 8 or 18 hours (Figure 3.7). Thus, 3 hours was the time point chosen for future analyses of the release of HSPs from C2C12 myotubes

3.3.4 The effect of TNF-α treatment on the HSP content of media

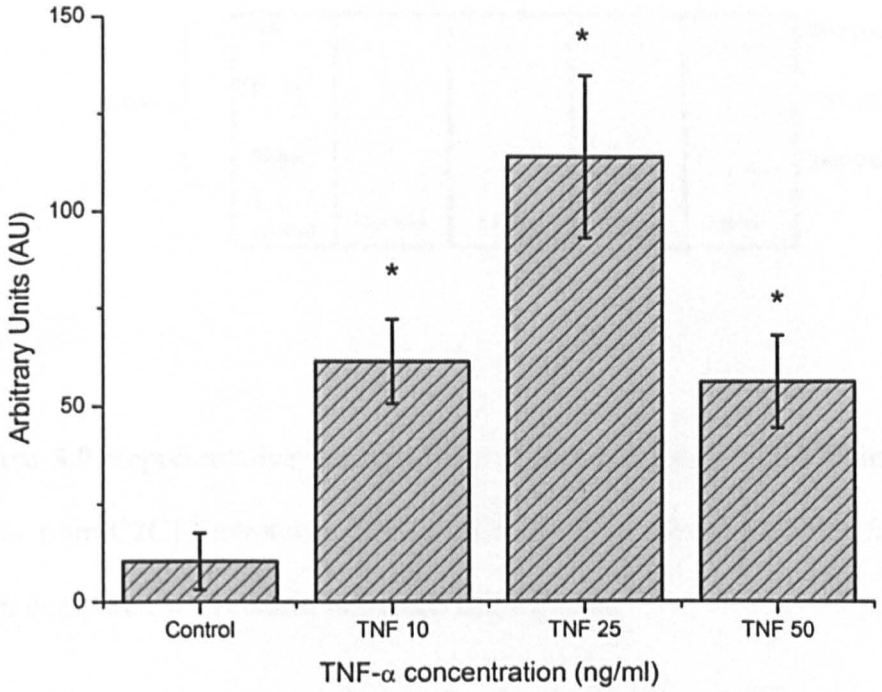


Figure 3.8 HSP60 content of cell culture media from C2C12 myotubes at 3 hours following treatment with 10, 25 or 50ng/ml of TNF-α compared with control un-treated cells. Data are presented as mean ± SEM (n=5); (*p≤0.05 compared with control untreated samples).

Data in Figure 3.9 show significant release of HSP60 from C2C12 myotubes when treated with 10, 25 or 50ng/ml TNF-α compared with the untreated control (Figure 3.8).

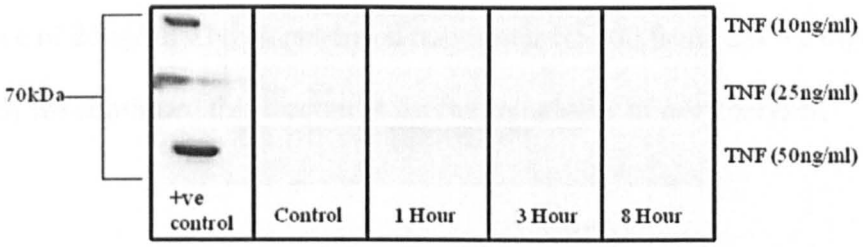


Figure 3.9 Representative western blots showing levels of HSP70 in cell culture media from C2C12 myotubes treated with 10, 25 or 50ng/ml TNF-α for 1, 3 and 8 hours compared with control untreated C2C12 cells.

Data shows no detectable levels of HSP70 present in the cell culture media in the un-treated controls and in response to TNF-α treatment at 1, 3 and 8 hours (Figure 3.9). HSP10 and HSC70 were also undetectable (Data not shown).

3.3.5 Effect of blocking golgi-mediated secretion on HSP60 release from myotubes at 3 hours following treatment with TNF-α

Since a dose of 25ng/ml TNF-α produced maximum HSP60 from C2C12 myotubes (Figure 3.6) we continued this treatment for the remainder of our analyses.

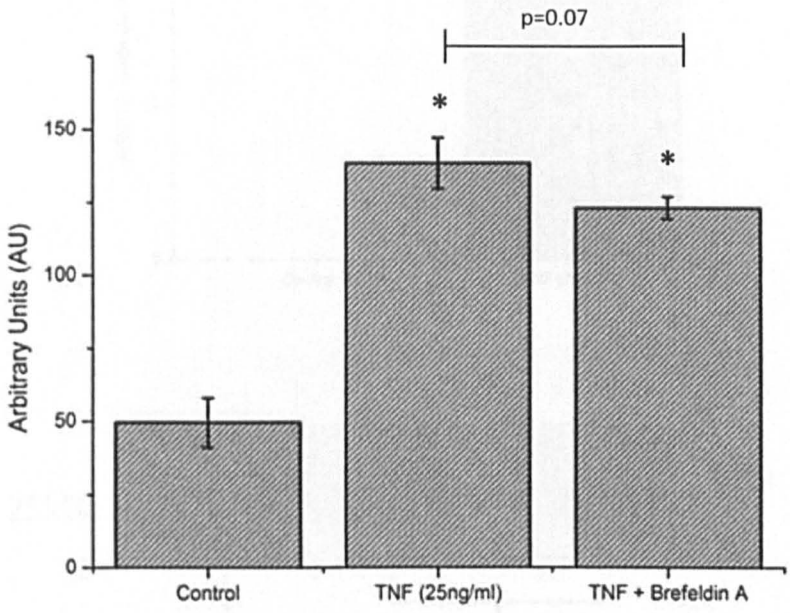


Figure 3.10 Effect of pre-treatment of C2C12 myotubes with Brefeldin-A (BFA) on HSP60 release from C2C12 following exposure to TNF-α (25ng/ml) for 3 hours. Data are presented as mean ± SEM (n=5),*p≤0.05 compared with untreated control cell culture media).

Pre-treatment of C2C12 myotubes with BFA had no significant effect on the release of HSP60 following treatment with TNF-α (Figure 3.10).

3.3.6 Examination of the release of exosomes from C2C12s myotubes following TNF-α treatment

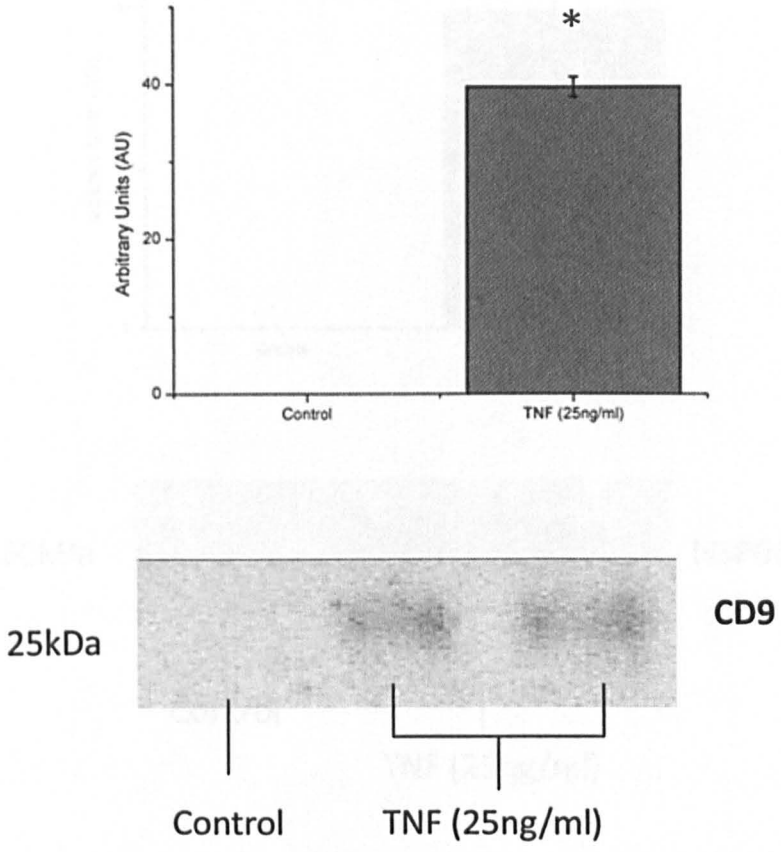


Figure 3.11 CD9 content of the exosomal fraction from C2C12 myotubes at 3 hours following treatment with 25ng/ml TNF-α. Data are presented as mean ±SEM (n=4) *p≤0.05 compared with control untreated cells.

The total protein determined in the exosome fraction from control cells was undetectable and CD9 was also undetectable in this fraction. In contrast, significant levels of CD9 were detected in the exosomal fraction from C2C12 myotubes treated with TNF-α for 3 hours, indicating that TNF-α induced exosome release from C2C12 myotubes (Figure 3.11).

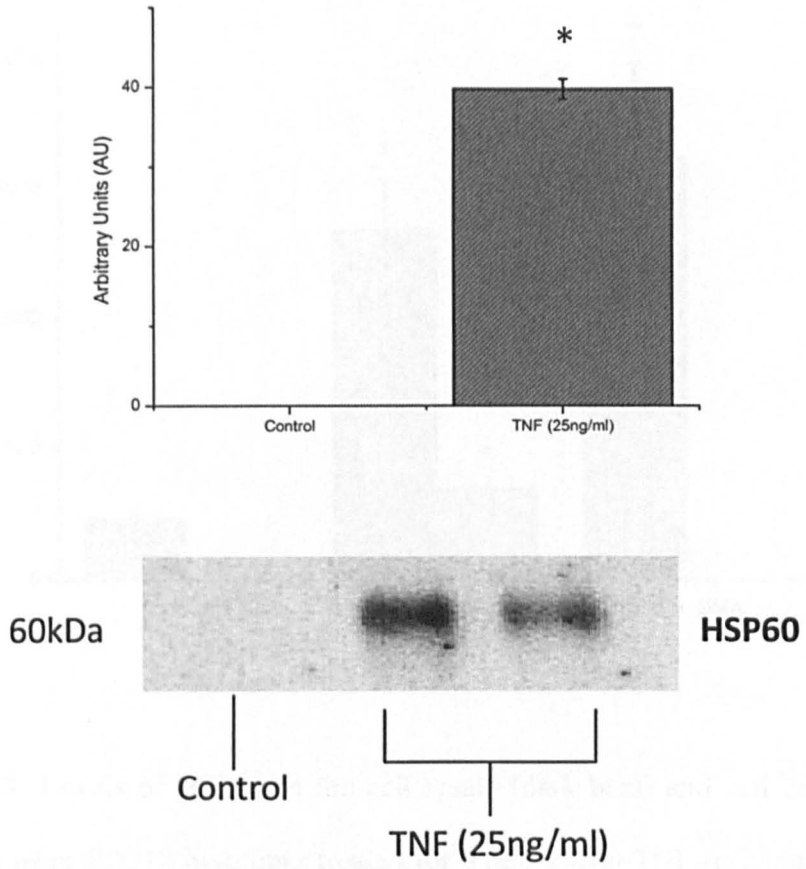


Figure 3.12 HSP60 content of the exosomal fraction of C2C12 myotubes treated for 3 hours with 25ng/ml TNF-α. Data are presented as mean ±SEM (n=4) *p≤0.05 compared with untreated control cells.

Significant levels of HSP60 were detected in the exosomal fraction from media of C2C12 myotubes treated with TNF-α (25ng/ml). No detectable levels were present in the equivalent fraction from untreated cells; this is most likely due to the lack of exosome released from untreated cells, as the protein content of this fraction was undetectable. These data indicate that TNF-α induced HSP60 release from C2C12 myotubes is associated with exosome release (Figure 3.11 and 3.12).

3.3.7 Effect of blocking the exosomal pathway on eHSP content

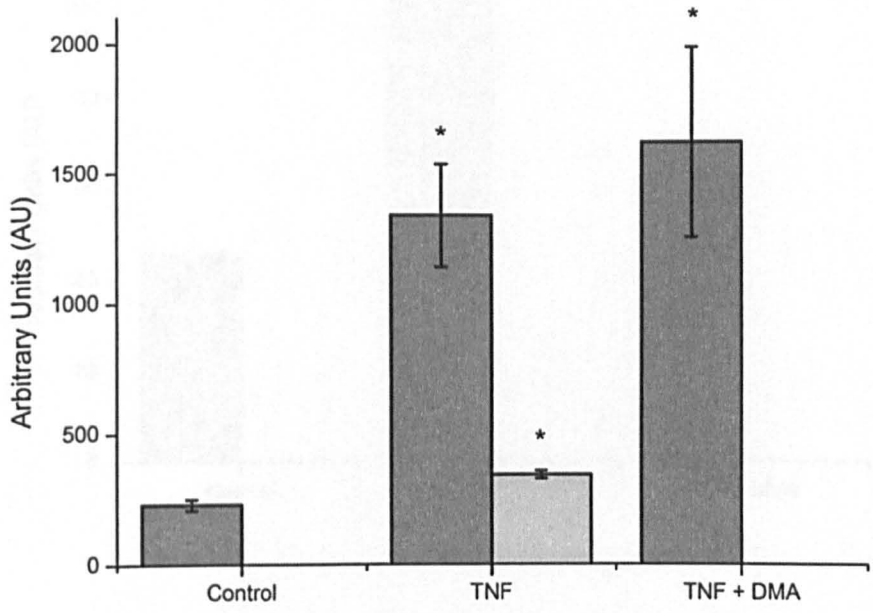


Figure 3.13 Levels of HSP60 in the cell lysate (dark bars) and cell culture media (light bars) from C2C12 myotubes treated for 3 hours with TNF-α (25ng/ml) with or without 1hr pre-treatment with dimethylamiloride (DMA). Data are presented as mean ± SEM (n=6) *p≤0.05 compared with untreated control cells. Samples were analysed on the same western blot to allow comparison of levels in the cell lysate and cell culture media.

Data show significant release of HSP60 from C2C12 myotubes in response to treatment with TNF-α. Pre-treatment with DMA to block exosomal transport had no effect on intracellular content of HSP60 but ablated TNF-α-induced HSP60 release from C2C12 myotubes. TNF-α induced the release of 20% of the total HSP60 content, (Figure 3.13) after 3 hours of treatment.

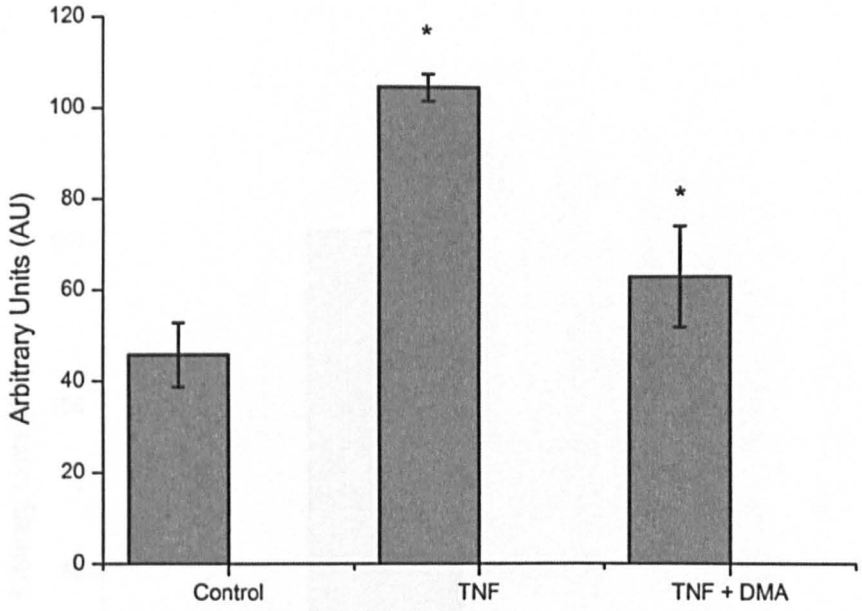


Figure 3.14 Levels of HSP70 in the cell lysate (dark bars) and cell culture media (light bars) from C2C12 myotubes treated for 3 hours with TNF-α (25ng/ml) with or without 1 hour pre-treatment with dimethylamiloride (DMA). Data are presented as mean ± SEM (n=6) *p≤0.05 compared with untreated control cells.

Data show significant elevation in intracellular levels of HSP70 in C2C12 myotubes treated with TNF-α as shown previously. No detectable levels of HSP70 were present in the cell culture media, prior to or following treatment with TNF-α. Pre-treatment of C2C12 cells with DMA resulted in a significant decrease in intracellular HSP70 levels compared with C2C12 myotubes treated with TNF-α alone (Figure 3.14). HSP10 and HSC70 were not examined in this fraction as no release of these proteins was previously seen in response to treatment with TNF-α.

3.3.8 Does up-regulation of intracellular HSPs by heat treatment invoke release?

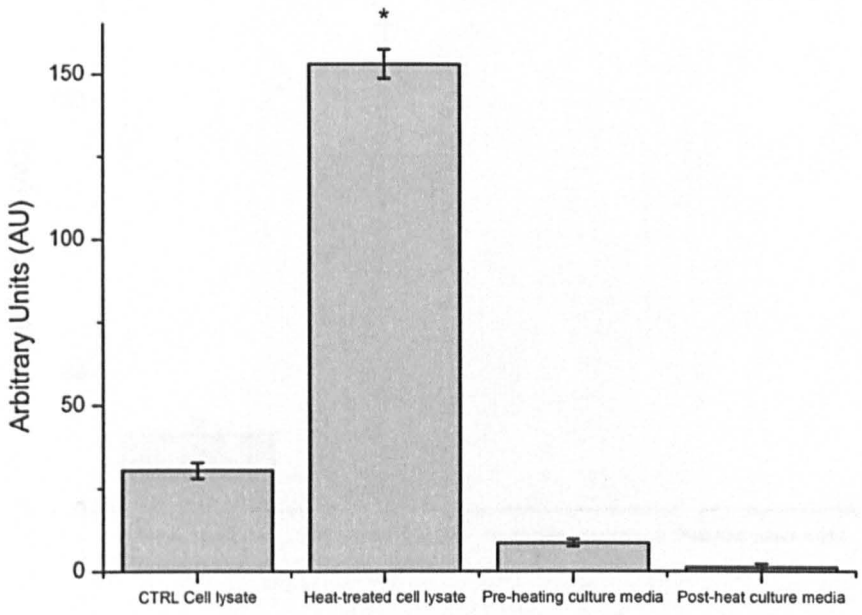


Figure 3.15 HSP60 levels in control C2C12 myotubes , heat-treated myotubes and in the media of control and heat treated myotubes. Data are presented as mean ±SEM (n=4) *p≤0.05.

Heat treatment of C2C12 myotubes resulted in a significant increase in intracellular content of HSP60 compared with unheated control cells. In contrast to the treatment with TNF-α heat treatment of C2C12 myotubes did not result in signficant release of HSP60 at 24 hours post treatment (Figure 3.15), when the intracellular HSP60 content was significantly elevated to a similar level to that seen following treatment of C2C12 myotubes with TNF-α (Figure 3.6).

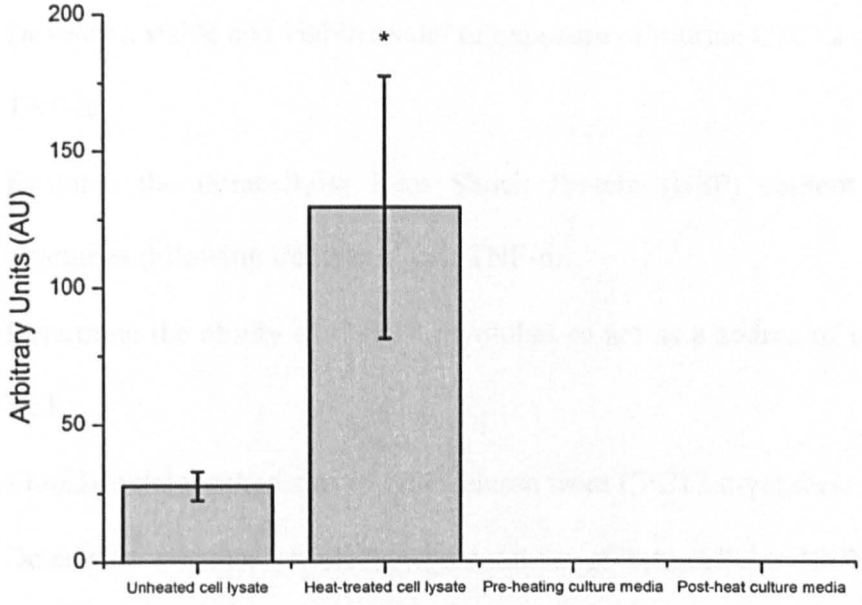


Figure 3.16 HSP70 levels in control C2C12 myotubes , heat-treated myotubes and in the media of control and heat treated myotubes. Data are presented as mean ±SEM (n=4) *p≤0.05.

Heat treatment of C2C12 myotubes resulted in a significant increase in intracellular content of HSP70, compared with control cells at 24 hours post-treatment. However, heat treatment of C2C12 myotubes did not result in signficant release of HSP70 into the cell culture media (Figure 3.16).

3.4 DISCUSSION

The aims of the study were to:

- Develop a stable and viable model of exposure of murine C2C12 myotubes to TNF-α.
- Examine the intracellular Heat Shock Protein (HSP) content of C2C12 myotubes following treatment with TNF-α.
- Determine the ability of C2C12 myotubes to act as a source of extracellular HSPs.
- Elucidate the mechanisms of HSP release from C2C12 myotubes.
- Determine whether physical augmentation of intracellular HSPs results in modified HSP release from C2C12 myotubes following hyperthermia, thus identifying whether changes in intracellular HSPs drive eHSP levels.

3.4.1 Development of a stable and viable cell culture model of inflammation

TNF-α is a potent inducer of catabolism and atrophy in skeletal muscle (Li et al., 2005). In addition high serum levels of TNF-α have a well defined role in the poor outcome associated with cachexia in critically ill septic patients (Saini et al., 2006). The decision to use TNF-α as a model of inflammation was due to the well characterised role of TNF-α as a master regulator of cytokine production, whereby it is responsible for propagating the elevation of a large array of cytokines (Chen and Goeddel, 2002). Furthermore, the distinct signalling pathways of TNF-α are well defined.

It was crucial to develop a non-necrotic and non-apoptotic system of exposure of muscle cells to inflammation, to exclude the role of necrotic cell lysis release of HSPs by TNF-α. Concentrations of TNF-α in the range of 10-50ng/ml have been shown to induce a cachectic response in C2C12 myocytes (Guttridge et al., 2000, Langen et al., 2001) and upregulation of inflammatory genes and receptors in muscle (Bhatnagar et al., 2010). Use of TNF-α in this controlled manner allows the determination of the role of TNF-α in production and release of HSPs by skeletal muscle cells.

Examination of cellular necrosis, using trypan blue staining and light microscopy showed no significant loss of cellular viability over a range of doses of TNF-α, for up to 8 hours of treatment (Figure 3.3). Similarly, using LIVE/DEAD calcein-AM/ethidium staining, (Figure 3.1 and 3.2) no significant loss of cellular viability was observed. No change in fibre size or morphology was evident under light microscopy. The effect of chronic exposure to TNF-α and its effect on cellular apoptosis is well described, however, this was not detectable by light microscopy alone. Biochemical analysis of cleaved caspase-3, a pivotal apoptosis marker in muscle (McArdle et al., 1999) by western blotting showed no detectable levels of caspase-3 for up to 8 hours following treatment with TNF-α (Figure 3.4). From these data it was concluded that the levels of TNF-α used to treat C2C12 myotubes did not result in any changes in cellular viability, by either necrosis or apoptosis.

3.4.2 The effect of TNF-α on HSP content of C2C12 myotubes and release of HSPs into the cell culture media

The main aim of this study was to determine whether treatment of C2C12 myotubes with TNF-α induced a stress response and subsequent release of HSPs from muscle

cells. An important consideration was to determine the stability of HSPs in a cell culture environment. Typically, these conditions deviate from an *in vivo* setting considerably, in particular oxygen saturation is markedly higher in a cell culture environment and released proteins may be degraded in the cell culture media. Understanding the degradation characteristics of HSPs is crucial to the interpretation of data examining HSP release from C2C12 myotubes.

The concentration of HSP70 in conditioned media did not change significantly after 3 hours of incubation (Figure 3.7), however, a significant reduction in the concentration of HSP70 was seen after 8 hours of incubation and a further reduction observed at 18 hours. The main cause of this loss of HSP70 protein is likely to be degradation as a result of the presence of proteases released from the C2C12 myotubes into the conditioned media, in addition to the stability of the HSP70 protein at 37°C. Therefore on this basis it was concluded that HSPs released from cultured cells and residin in the media are stable for up to 3 hours, indicating that any measurements made of released HSPs before this time point are valid but after this timepoint may not reflect the actual quanitities of protein originally released by the cells.

TNF-α is a potent inflammatory agent, with widely characterised roles influencing immunity, protein synthesis and degradation as well as apoptosis (Li et al., 2005); (Lin et al., 2007). The induction of cellular stress by TNF-α is well documented (Hardin et al., 2008). However, the effect of TNF-α on the stress response in skeletal muscle is poorly defined. Using the viable model of inflammation developed in the initial stages of this study, the heat shock protein response was examined by determining changes in HSP protein content of C2C12 myotubes following treatment with TNF-α (Figure 3.5). TNF-α treatment resulted in a significant rise in

intracellular HSP10, HSP60, and HSP70 (Figure 3.6) in treated compared with untreated cells. Although the reason for such HSP induction is unknown, data suggest a role of HSPs in cytoprotection in response to stress, possibly by the well characterised anti-apoptotic function of HSPs (Meldrum et al., 2001). There was approximately a 3-fold increase in HSP60 protein content of cells and a 4-fold increase in HSP10 content, when compared with untreated control cells, independent of TNF-α dose following 3 hours of exposure. TNF-α treatment also induced a dose dependent increase in HSP70 levels. HSP60 and HSP10 form a chaperonin complex in the mitchondria (Fink, 1999), such up-regulation suggests a cytoprotective role in the mitochondria as demonstrated in several models such as cardiac muscle following ischemic injury (Dillmann, 1999). Recent data has suggested that TNF-α acts to increase ROS production by mitochondria (Reid and Moylan, 2011) although the mechanisms by which this occurs are poorly understood. These data are in keeping with this proposal. In contrast, muscle cells showed no evidence of increase in HSC70 content in response to TNF-α treatment regardless of concentration (Figure 3.5). This lack of response is perhaps as a result of the overall nature of HSC70, whereby it is widely accepted to play an important role as a chaperone in unstressed cells during protein synthesis but is less responsive to stress (Hartl, 1996, Fink, 1999).

Significant levels of HSP60 were detected in the cell culture media from C2C12 myotubes in response to treatment with TNF-α (Figure 3.8). A 5-fold increase in extracellular HSP60 (eHSP60) was observed when compared with untreated control cells. These data (Figure 3.8) have demonstrated for the first time the release of HSP60 from viable skeletal muscle in response to TNF-α treatment. No other HSPs were detected in the cell culture media suggesting that HSP60 is released in a

specific manner from C2C12 myotubes (Figure 3.8). These data support studies in other tissues which have shown HSP60 to be released from viable cells and reside in the extracellular environment, when examining the effect of celluar stress on cardiac myocytes (Gupta and Knowlton, 2007) and in the circulation of patients with sepsis (Wheeler et al., 2007). The specific release of HSP60 suggests that there may be a discrete mechanism in place which perhaps allows this protein to perform the widely described function of eHSPs as "danger-signals" (Asea, 2005). The representative western blot image (Figure 3.9) shows no detectable levels of HSP70 present in the extracellular media following TNF-α exposure for up to 8 hours. HSP70 is widely described as a key eHSP and the role of eHSP70 in immuno-modulation as a chaperokine in a CD14 dependent manner (Asea et al., 2000b) is well defined (Asea, 2006, Asea, 2008b). The presence of HSP60 and not HSP70 in the cell culture media and in purified exosomes suggests a specific mechanism for HSP60 release. Previous data in this chapter shows HSP70 to be stable in culture for up to 3 hours, with only marginal degradation (Figure 3.4) – therefore stability of the HSP70 protein *in vitro* does not appear to be responsible for the lack of detection in these studies.

HSP60 is primarily mitochondrially associated (Hood et al., 2003), however, studies examining cardiac muscle injury have shown that HSP60 migrates to the plasma membrane with externalisation into the extracellular space via a non-classical pathway (Gupta and Knowlton, 2007) and data shown here supports the specific externalisation of HSP60 analogous to that seen in cardiac muscle. The ability of skeletal muscle to secrete HSP60 to act as a chaperokine suggests skeletal muscle may act as a significant source of circulating eHSP60 in pathophysiological conditions. Furthermore, recent data have shown mitochondrial associated components termed damage-associated molecular patterns (DAMPs) are released

from adult muscle into the systemic mileu and are capable of activating polymorphonuclear cells (PMNs) and contribute to an overall systemic inflammatory cascade (Zhang et al., 2010a).

Induction of specific mitochondrial stress in *Caernorhabditis elegans* has demonstrated an additional mitochondrial stress response in a distal tissue. The induction of an unfolded protein response (UPR) in the mitochondria of *c.elegans* presented pertubations in the intestine, the cellular messengers involved in this process are not fully understood, they have however been termed mitokines – specific mitochondria-associated molecules which have cytokine-like signalling properties in the systemic environment (Durieux et al., 2011). Data presented in this chapter are in agreement with this work and suggest eHSP60 may be acting as a mitokine.

The data presented here provide a new perspecitve on recent findings that mitochondrial associated molecules are released and could have potential systemic signalling functions.

3.4.3 Mechanisms of release of HSPs from Skeletal Muscle

The data presented here show a clear and specific release of HSP60 from viable C2C12 myotubes following treatment with TNF-α, independent of any other members of the HSP family. However, the means by which this specific release occurs is of significant interest. A classical and ubiquitous means of protein secretion applicable to the majority of cell/tissue lineages is the golgi mediated pathway, whereby translated protein is sorted and modified by the golgi complex allowing release via the plasma membrane.

To determine the specific pathways for HSP60 release from skeletal muscle cells in response to TNF-α, golgi-mediated export was blocked using a well characterised blocking agent, Brefeldin A (Gupta and Knowlton, 2007). Brefeldin A causes accumulation of protein in the endoplasmic reticulum, preventing transport to the golgi apparatus (Nebenfuhr et al., 2002).

Western blot analysis for the presence of HSP60 in cells pre-treated for 1 hour with Brefeldin A (BFA) and then treated with TNF-α for 3 hours showed no effect of BFA on the levels of HSP60 in the media compared with the cells treated with TNF-α alone (Figure 3.10). These data are supported by the fact that the well characterised structure and sequence of HSP60 lacks a classical leader sequence in the peptide structure which is normally required for golgi targeted secretion (Asea, 2007). In light of this, research in cardiac cells has shown release of HSP60 via a non-classical exosomal pathway, where HSP60 is localised within exosome vesicles from cardiac muscle (Gupta and Knowlton, 2007). Exosomes were isolated from cell culture supernatants from TNF-α treated C2C12 myotubes using a standard multi-step ultracentrifugation prototcol (Valadi et al., 2007). Western blot analysis of the exosome fraction for the presence of HSP60 and CD9 (a known classical exosome marker); (Valadi et al., 2007, Eldh et al., 2010) showed signficant levels of both HSP60 and CD9 targets in the exosomes following TNF-α treatment (Figures 3.11 & 3.12). These data show for the first time that skeletal muscle myotubes can release exosomes in response to treatment with TNF-α and these exosomes contain HSP60. Moreover, this suggests that, for C2C12 myotubes to release exosomes a stimulus is required since no exosomes were detected in media from the untreated control cells.

The precise intra-cellular source of the HSP60 is unclear, whether it is newly synthesised HSP60 in response to the TNF-α treatment that is re-directed from the cytosol, or export of HSP60 already located within the mitochondria. One hypothesis is that there is a population of HSP60 that resides on or near the plasma membrane of the cell, and this is described in detail in cardiac cells (Gupta and Knowlton, 2007, Lin et al., 2007) and it hypothesised that this population is internalised into exosomes by inward budding of the plasma membrane and the subsequent formation of the late endosome and multivesicular bodies (Figure 1.7).

Studies have shown that undifferentiated C2C12 myoblasts secrete exosomes contanining mitochondrial DNA and mRNA (Guescini et al., 2010). Exosomes have been widely described immunostimulatory particles (Johnstone, 2006) and a novel means of genetic transfer (Valadi et al., 2007). We analysed exosomes from C2C12 myotubes for additional mitochondrial components, however, none were detectable.

To identify that exosomes are the major route of HSP60 release from skeletal muscle, C2C12 myoblasts were pre-treated with Dimethyl Amiloride (DMA) a known exosome blocker, through disruption of calcium signalling (Gupta and Knowlton, 2007, Savina et al., 2003). DMA blocks the Na^+/H^+ and Na^+/Ca^{2+} pumps (Savina et al., 2002, Savina et al., 2003). Exosome release has been shown to be dependent on Ca^{2+} influx into the cytosol and complete inhibition of exosome release was shown when K562 cells were treated with DMA prior to challenge with calcium ionophore A21387 (Savina et al., 2003).

Pre-treatment of C2C12 myotubes with DMA for 1 hour prior to 3 hours of TNF-α treatment resulted in significantly reduced levels of HSP60 in the extracellular media (Figure 3.13). These data suggest that HSP60 is localised within the exosomal

vesicles produced by C2C12 myotubes following TNF-α treatment, and this appears to be the predominant mechanism of HSP60 release. Furthermore, this is the first study to show TNF-α to have exosome stimulating capability in muscle cells in culture.

3.4.4 Up-regulation of intracellular HSPs by hyperthemia, and the impact on HSP release.

Exposure of cells to hyperthemia is a well defined method of elevating HSP content of cells (Maglara et al., 2003). C2C12 myotubes heated at 42°C for 1 hour showed significantly elevated intracellular levels of both HSP60 and HSP70 when compared with the unheated control; however, in both cases no significant changes in extracellular HSP content were detectable at 3 hours post-heating (Figure 3.15 & 3.16). These observations are in contrast to recent *in vivo* work which demonstrated hyperthermia induced in rats caused elevations in circulating levels of HSP70 (Bathaie et al., 2010). We can therefore hypothesise that release of HSP70 into circulation in response to hyperthermia is from a non-muscle source.

The release of exosomes has been described in response to a whole variety of stimuli from mechanical, cytokine-induced and in the presence of reactive oxygen species (ROS) (Johnstone, 2006) and perhaps release demonstrated here is part of a first messenger response to communicate to neighbouring cells and tissues of an impending insult.

3.5 SUMMARY

In summary, work in this chapter has developed a stable cell culture model of inflammation. TNF-α treatment induced an increase in the intracellular levels of HSPs in C2C12 myotubes and HSP60 was primarily and specifically released from C2C12 myotubes by an active process of exosome release. Data in this chapter shows for the first time the ability of viable skeletal muscle to selectively externalise HSP60 via specific non-classical means (exosome vesicles).

CHAPTER 4

THE EFFECT OF TNF-α TREATMENT ON MYOKINE RELEASE FROM C2C12 MYOTUBES

4.1 Introduction

Research pioneered by Pedersen and colleagues in Copenhagen has described the capability of skeletal muscle to act as a major source of cytokines (Ostrowski et al., 2000).

4.1.1 The role and function of myokines

Release of IL-6 by skeletal muscle in response to exercise in humans (Pedersen et al., 2003) and in rats showed significant increase in mRNA levels of IL-6 following lengthening and shortening contractions of the *gastrocnemius* muscle compared with the contralateral control muscle (Jonsdottir et al., 2000). It was originally suggested that this observed elevation in circulating cytokines was as a result of local muscle damage, and the subsequent infiltration of immune cells to the site. However, data suggests that circulating monocytes were not the source of elevated plasma IL-6 post-exercise (Starkie et al., 2001). Further studies demonstrated that IL-6 was expressed by and released from contracting skeletal muscle. Exercise resulted in increased IL-6 mRNA levels in human muscle and in plasma levels of IL-6 (Steensberg et al., 2002). Further work has demonstrated a fibre-type specificity to cytokine production, whereby IL-6 protein levels were higher in type I compared with type II fibres from healthy males, furthermore TNF-α and IL-18 were only expressed in type II muscle fibres (Plomgaard et al., 2005).

Since these studies, additional cytokines have been identified which are produced by skeletal muscle. Interleukin 8 (IL-8) mRNA a potent neutrophil chemoattractant was shown to be elevated in muscle biopsies taken from humans following exercise (Akerstrom et al., 2005), suggesting a local role for muscle derived IL-8 in angiogenesis.

Interleukin 15 (IL-15) has also been shown to be derived from skeletal muscle; clear elevation in IL-15 mRNA was seen in muscle in response to exercise in humans (Nielsen et al., 2007). IL-15 has been demonstrated to attenuate white adipose tissue deposition in rats (Carbo et al., 2001) and numerous studies have reported IL-15 as key player in the interaction between muscle and adipose tissue.

Overall, data suggest that exercise induces a system of low-grade inflammation, mediated at least in part by muscle which is in turn physiologically beneficial. Up-regulation of IL-6 in muscle by exercise has been the main area of focus of previous studies indicating that IL-6 has both pro- and anti-inflammatory properties. Exercise-induced elevated circulating IL-6 is accompanied by elevated levels of IL-1ra and IL-10, key anti-inflammatory agents (Steensberg et al., 2003). An anti-inflammatory response is proposed to be activated by IL-6 which has been shown to suppress TNF-α levels *in vitro* (Mizuhara et al., 1994). Furthermore *in vivo* studies have demonstrated that IL-6 infusion in humans ablates endotoxin induced production of TNF-α (Starkie et al., 2003). Conversely, elevated systemic levels of IL-6 have been measured in aged individuals, and association was demonstrated between circulating IL-6 levels and loss of muscle mass and increased morbidity (Ferrucci et al., 2002); this is somewhat paradoxical to the beneficial role of IL-6 reported in the previously mentioned exercise models (Steensberg et al., 2003) and remains unexplained. A summary of the currently described myokines in the field can be found in Table 4.1.

Myokine	Treatment	Detected	Study
IL-6	Exercise	mRNA in human muscle biopsy. Protein in plasma.	Plomgaard et al. (2005) Pedersen & Fischer (2007)
IL-7	Differentiation	Primary human muscle cells.	Haugen et al. (2010)
IL-8	Exercise	mRNA in human muscle biopsy.	Akerstrom et al. (2005)
IL-15	Exercise Transfection	mRNA in muscle tissue. Protein in plasma	Pedersen et al. (2007) Quinn et al. (2009)
IL-18	Exercise	mRNA & protein in human muscle biopsy	Plomgaard et al. (2005)
TNF-α	Exercise	mRNA & protein in human muscle biopsy	Plomgaard et al. (2005)

Table 4.1 Currently published research on cytokines detected in and potentially released from skeletal muscle.

4.1.2 The role of muscle in the cytokine response during sepsis

During sepsis the increased serum levels of IL-6 occurs in response to the main initiators of inflammation TNF-α and IL-1β; following which an anti-inflammatory cascade is initiated with the elevation of IL-1ra, soluble TNF receptor (sTNF-R) and IL-10 (Akira et al., 1993).

In the critically ill it is the unique interaction between pro- and anti-inflammatory pathways that is a defining characteristic, more than often resulting in an unfavourable outcome. Therefore the possibility that IL-6 may act as a controller of the anti-inflammatory response, albeit during exercise, is an exciting point of interest in understanding the complex interactions of the inflammatory cascade.

Thus, skeletal muscle functions as an endocrine organ. The research so far has not investigated the effect of chronic inflammation on myokine production *in vivo*.

Studies have shown that TNF-α treatment results in the up-regulation of mRNA levels of IL-6 and IFN-γ in C2C12 myotubes (Alvarez et al., 2002a) with additional changes in anti-inflammatory cytokine mRNA of IL-4, IL-9, IL-10, IL-15 and TGF-β1 (Alvarez et al., 2002b). In C2C12 myotubes work has been extended recently to demonstrate changes in approximately 5000 genes in response to TNF-α treatment in C2C12 myotubes. The study used a microarray approach to show the up-regulation of numerous genes involved the inflammatory cascade, apoptosis, cellular proliferation and differentiation in response to TNF-α treatment (Bhatnagar et al., 2010).

It is clear that TNF-α has a profound effect upon regulating gene expression in skeletal muscle. Data in Chapter 3 demonstrated that skeletal muscle can release HSP60 primarily in exosomes, in response to treatment with TNF-α. Furthermore, exosomes released from C2C12 myoblasts contain a plethora of proteins, including chaperones and signal transduction proteins (Guescini et al., 2010). The role of exosomes as immunomodulators is well defined (Asea et al., 2000b), therefore, there may be a link between TNF-α induced inflammatory augmentation in skeletal muscle and the release of myokines as part of exosomes or through classical exocytic means (Lightfoot et al., 2009). We hypothesise that the treatment of C2C12 myotubes with TNF-α will result in increased synthesis and release of myokines and this will occur via both golgi-meditated and exosome pathways.

4.1.3 AIMS

The aims of the study were to:

- Investigate the effect of TNF-α on changes in inflammatory gene expression in C2C12 myotubes.
- Determine the effect of TNF-α on the release of cytokines (myokines) from skeletal muscle
- Investigate the pathways of release of cytokines through the examination of the golgi-mediated and exosomal pathway.

4.2 METHODS

4.2.1 The effect of TNF-α on inflammatory gene expression in C2C12 myotubes.

C2C12 myotubes were grown in culture as described in Section 2.1. Myotubes were treated with TNF-α in FCS depleted media, as described in Section 2.1.2. A does of 25ng/ml of TNF-α was used throughout, as it yielded a peak release of HSP60 shown in Chapter 3. Cells were harvested and lysates were subject to RNA isolation as described in Section 2.2.2, followed by first strand synthesis (Section 2.2.1.3) and subsequent PCR array analysis (SAbiosciences, Maryland, USA; Section 2.4). Real time PCR was used to examine the changes in gene expression of IL-6 (not present on the qPCR array) as described in Section 2.4. Genes of interest reporting a threshold cycle (Ct) of greater than 35 were discounted from analysis. Ct values greater than 35 typically indicate that the expression of the gene was low or not present, therefore any discernable difference between control and experimental samples was not possible.

4.2.2 The effect of TNF-α on the release of myokines from C2C12 myotubes.

C2C12 myotubes were grown in culture as described in Section 2.1. Myotubes were treated with TNF-α in FCS-depleted media, as described in Section 2.1.2. Concentrations of cytokines in cell culture media were determined using Bioplex multi-bead analysis as described in Section 2.3.5. Cytokine levels were normalised to total cellular protein quantified using the BCA assay as described in Section 2.3.1.

4.2.3 Examination of myokine release through golgi-mediated and exosomal pathways.

C2C12 myotubes were grown in culture as described in Section 2.1. Myotubes were treated with TNF-α in FCS-depleted media, as described in Section 2.1.2. Cells were pre-treated with BFA, DMA or vehicle control as described in Section 2.1.2. Cell culture media was harvested and subject to an acetone precipitation procedure as described in Section 2.1.3. Exosomes were isolated from cell culture media as described in Section 2.2.1. Myokines were detected in the exosomal and remaining non-exosomal fraction from cell culture media using Bioplex multi-bead analysis as described in Section 2.3.5. Process detailed in the schematic below (Figure 4.1). Cytokine levels were normalised to total cellular protein, quantified using the BCA assay as described in Section 2.3.1.

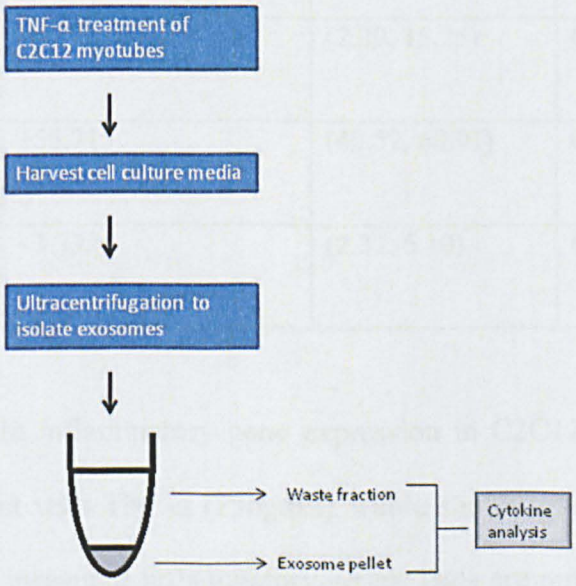


Figure 4.1 schematic of exosome isolation.

4.3 RESULTS

4.3.1 The effect of TNF-α on inflammatory gene expression in C2C12 myotubes.

Gene of Interest	Fold Change	95% CI	*p-value*
Complement component 3	+59.7141	(7.49, 111.94)	**0.042***
Chemokine (C-C motif) ligand 2 **(MCP-1)**	+34.296	(12.86, 55.74)	**0.048***
Chemokine (C-C motif) ligand 5 **(RANTES)**	+122.219	(114.47, 129.97)	**0.001***
Chemokine (C-C motif) ligand 7	+2.193	(0.36, 4.03)	0.231
Chemokine (C-C motif) ligand 9	+2.894	(0.86, 4.93)	0.093
Chemokine (C-X-C motif) ligand 1 **(KC)**	+9.849	(0.001, 21.44)	0.209
Chemokine (C-X-C motif) ligand 10 (IP-10)	+24.25	(18.51, 30.00)	**0.001***
Chemokine (C-X-C motif) ligand 11	+2.000	(1.48, 2.52)	**0.017***
Chemokine (C-X-C motif) ligand 5 (Ena-78)	+8.774	(2.29, 15.25)	0.091
Chemokine (C-X-C motif) ligand 9	+55.715	(42.52, 68.91)	**0.001***
Macrophage migration inhibitory factor	+3.732	(2.37, 5.10)	**0.019***

Table 4.2 Fold change in inflammatory gene expression in C2C12 myotubes at 3 hours following treatment with TNF-α (25ng/ml). Table shows up-regulation of 11 genes from a total of 84 measured inflammatory genes. Data are represented as fold change normalised to β-actin, alongside their 95% confidence interval (CI),* $p \leq 0.05$ (n=3).

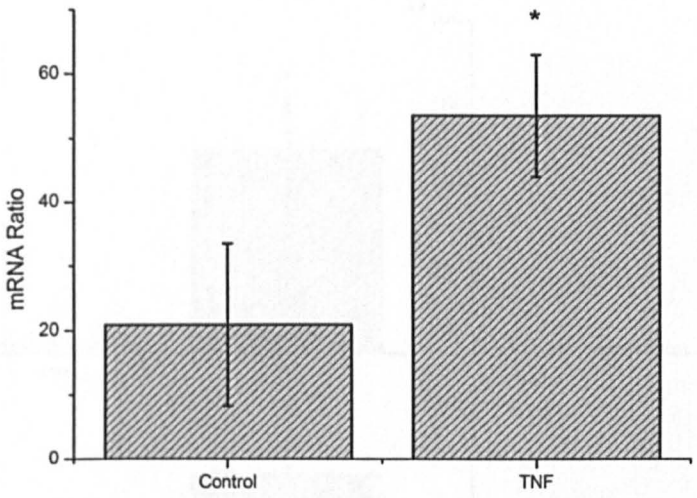


Figure 4.2 mRNA levels of IL-6 in control untreated C2C12 myotubes and in C2C12 myotubes treated with TNF-α. Data are presented as mean ± SEM (n=3) normalised to β-actin; *p≤0.05 compared with untreated control cells.

A significant increase was observed in IL-6 mRNA content of C2C12 myotubes treated with TNF-α when compared with untreated control cells (Figure 4.2).

4.3.2 The effect of TNF-α on myokine release from C2C12 myotubes

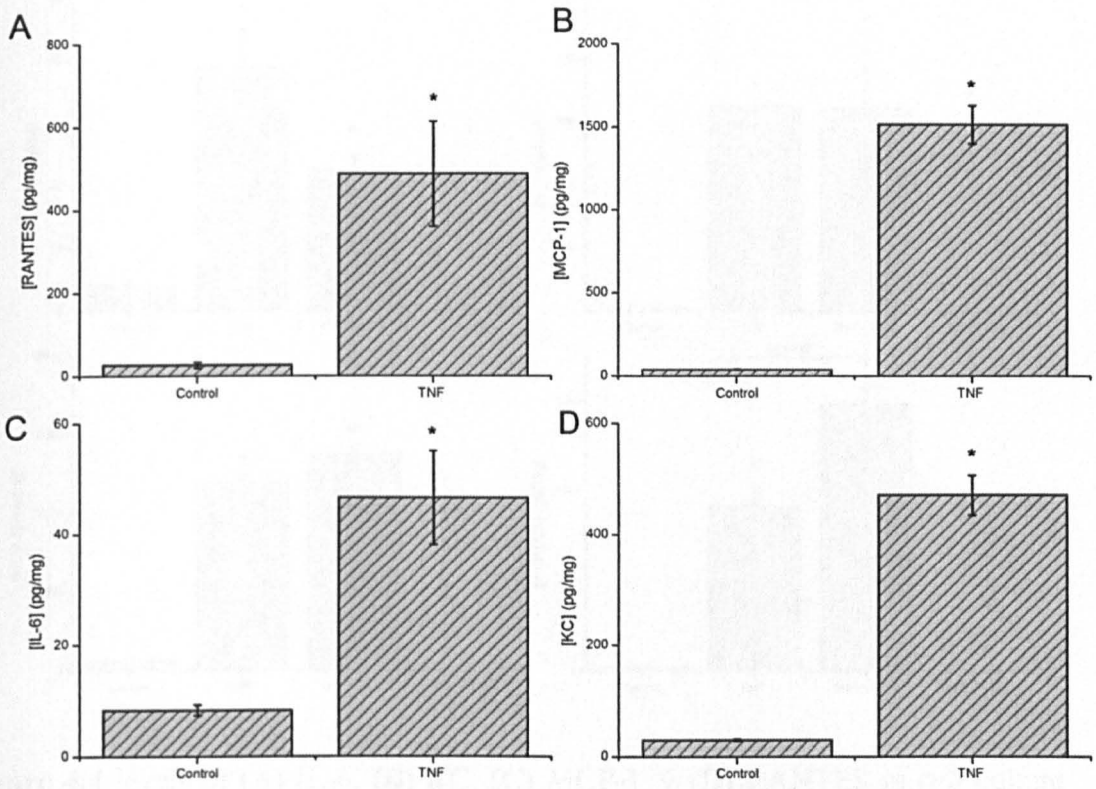


Figure 4.3 Levels of **(A)** RANTES, **(B)** MCP-1, **(C)** IL-6 and **(D)** KC released from C2C12 myotubes into the cell culture media at 3 hours following treatment with TNF-α (25ng/ml). Data are normalised to total cellular protein and presented as mean ±SEM (n=6)*p≤0.05 compared with untreated control cells.

Significant increase in media concentraion of RANTES, MCP-1, IL-6 and KC was observed from C2C12 myotubes at 3 hours following treatment with TNF-α (25ng/ml when compared with untreated control cells (Figure 4.3). No detectable levels of the following cytokines were found in the cell culture media in the control or in response to treatment with TNF-α: FGF-Basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, MIG & MIP1-α.

4.3.3 The cellular pathways involved in myokine release from skeletal muscle

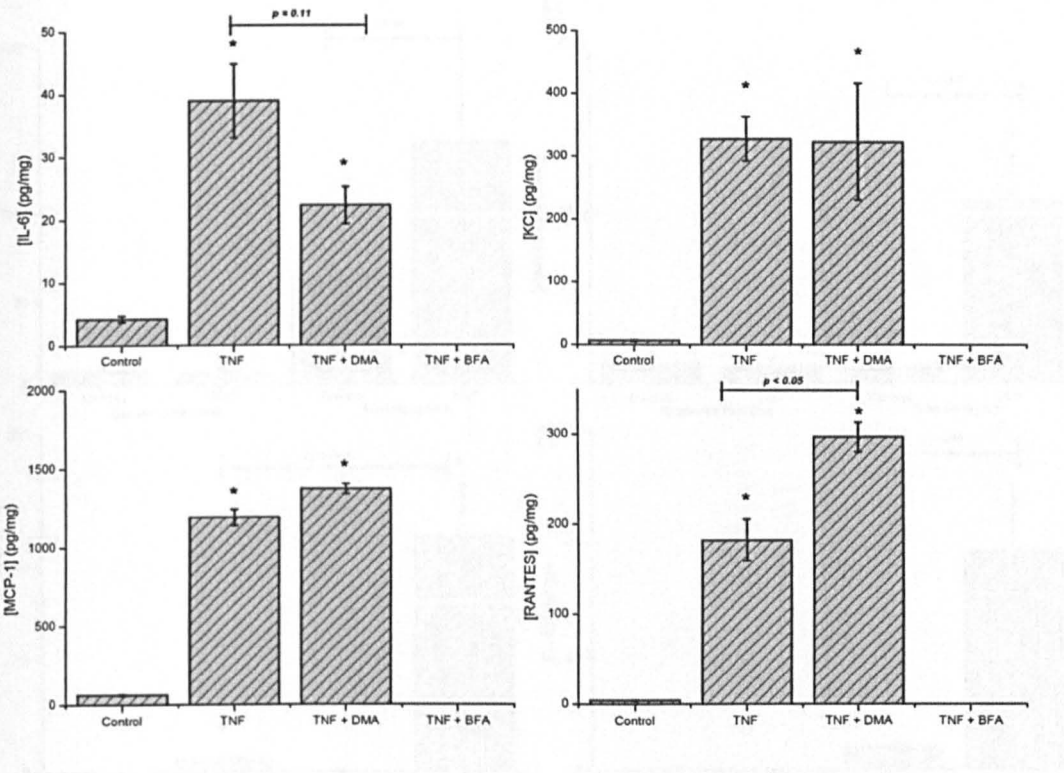


Figure 4.4 levels of **(A)** IL-6, **(B)** KC, **(C)** MCP-1 & **(D)** RANTES in cell culture media from C2C12 myotubes at 3 hours following treatment with TNF-α (25ng/ml). Cells were also pre-treated with either 17nM dimethyl amiloride (DMA) or 1μg/ml Brefeldin A (BFA) for 1 hour prior to treatment with TNF-α. Data are normalised to total cellular protein and presented as mean ±SEM (n=6) *p≤0.05 compared with untreated control cells.

Pre-treatment of C2C12 myotubes with DMA to inhibit exosomes release resulted in no significant change in the media concentration of KC and MCP-1 following treatment of myotubes with TNF-α. Pre-treatment with DMA caused an increase in the concentration of RANTES in media from C2C12 myotubes when compared with TNF-α treatment alone. Pre-treatment with BFA to inhibit golgi-mediated transport ablated TNF-α induced increase of all four cytokines in the media (Figure 4.4).

4.3.4 The myokine content of exosomes-derived from C2C12 myotubes

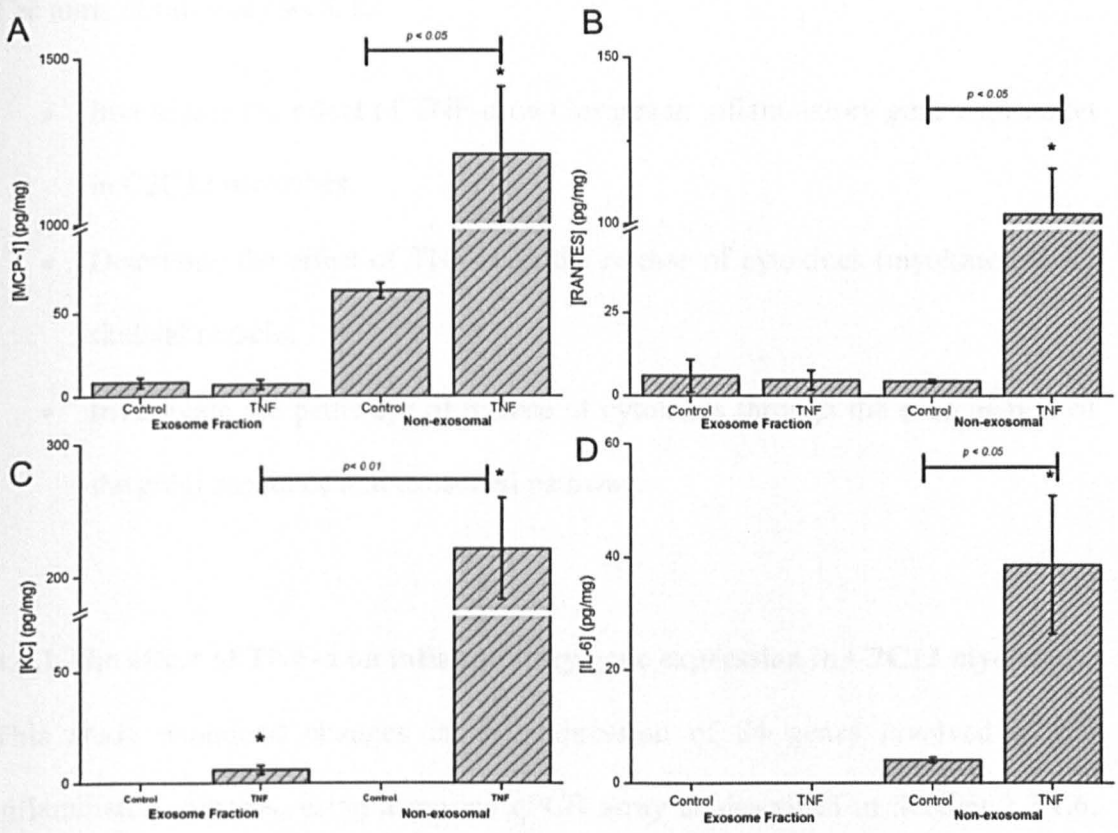


Figure 4.5 Levels of **(A)** KC, **(B)** MCP-1, **(C)** RANTES and **(D)** IL-6 in the exosomal and non-exosomal fraction from the cell culture supernatants in response to exposure to TNF-α (25ng/ml) for 3 hours. Data are normalised to total cellular protein and presented as mean ± SEM (n=3-5) *p≤0.05 compared with untreated control cells.

Examination of the exosomes purified from TNF-α-treated C2C12 myotubes demonstrated low or undetectable levels of KC, MCP-1, RANTES and IL-6. In contrast, significant levels of all four target cytokines were found in the remaining non-exosomal fraction from media of C2C12 myotubes treated with TNF-α, demonstrating that the release of cytokines from skeletal muscle cells was primarily non-exosomal (Figure 4.5).

4.4 DISCUSSION

The aims of the study were to:

- Investigate the effect of TNF-α on changes in inflammatory gene expression in C2C12 myotubes.
- Determine the effect of TNF-α on the release of cytokines (myokines) from skeletal muscle.
- Investigate the pathways of release of cytokines through the examination of the golgi-mediated and exosomal pathway.

4.4.1 The effect of TNF-α on inflammatory gene expression in C2C12 myotubes.

This study examined changes in the expression of 84 genes involved in the inflammatory process, using a murine qPCR array as described in Section 2.2.1.6. Significant up-regulation of gene expression occurred in 7 genes in myotubes response to treatment with a non-damaging level of TNF-α (Figure 4.1). There was no significant change in the remaining 77 genes analysed, the complete list can be seen in Section 2.4.

Up-regulation of the genes which encode MCP-1 (59-fold), RANTES (34-fold) and CXCL5 (8-fold) reflect the changes in gene expression seen in C2C12 myotubes in response to treatment with TNF-α reported recently (Bhatnagar et al., 2010). The changes in MCP-1 and RANTES expression are markedly higher than those reported by Bhatnagar and colleagues (2010); however this may be due to the difference in TNF-α dose used. The study reported in this chapter used a higher dose of 25ng/ml over a shorter timeframe compared with 10ng/ml over 18 hours – however, both doses are relevant (Guttridge et al., 2000, Stewart et al., 2004). Data clearly show

TNF-α treatment induced changes in inflammatory gene expression in skeletal muscle. Data describe novel up-regulation in gene expression for the following cytokines, C3 (59-fold), CXCL10 (24-fold), CXCL11 (2-fold), CXCL9 (55-fold) and MIF (3-fold) as well as increases in the previously reported cytokines CCL9 (2.1-fold), CXCL1 (9-fold), CCL7 (2-fold) and and 8-fold increase in CXCL5 (Bhatnagar et al., 2010).

Due to the absence of IL-6 on the gene array, qPCR analysis for the IL-6 gene, demonstrated a significant up-regulation of IL-6 mRNA in myotubes (2.5-fold) was seen following treatment with TNF-α. The up-regulation of IL-6 is consistent with the observation made by Alvarez et al. (2002a). However, the observed fold change in this chapter was markedly smaller than those observed in the Alvarez study. This is likely to be due to the supra-physiological levels of TNF-α (1000ng/ml) used by the investigators in that study (Alvarez et al., 2002a). What is more striking is that the study in this thesis, and the study of Bhatnagar et al. (2010) observed no change in anti-inflammatory cytokine mRNA, in particular IL-4, IL-10 and TGF-β1, which was described in the previous study (Alvarez et al., 2002a). The lack of observed change in anti-inflammatory genes suggests that skeletal muscle has potential to augment systemic inflammation as a pro-inflammatory organ through the up-regulation of pro-inflammatory genes. Understanding the effect of different stresses on the production of pro- or anti-inflammtory cytokines by muscle is critical to understanding the positive and negative effects of such stresses. The exercise induced anti-inflammatory response from muscle could have a role in striking a balance in instances of chronic inflammation.

Although the changes at the mRNA level are important in the interpretation of data, the functional aspect can only be demonstrated at the level of protein expression. The release of a broad cohort of inflammatory cytokines from skeletal muscle has important implications, particularly in terms of systemic inflammation. Moreover, this will provide insight into the contribution of myokines in the chronic systemic inflammation in the critically ill. Myokines may have a significant role to play in the chronic inflammatory cascade, either in terms of combating the initial infectious insult, or in exacerbating the profound inflammatory response that causes multiple organ failure.

4.4.2 The effect of TNF-α on the release of myokines from skeletal muscle.

To date, the only cytokines shown to be released from skeletal muscle are IL-6, IL-8, IL-15 and TNF-α (Ostrowski et al., 2000, Carbo et al., 2001, Akerstrom et al., 2005, Steensberg et al., 2002) and this work is based on different exercise models. There is currently a poor understanding of the effect of inflammation (TNF-α) on myokine production by skeletal muscle. The data in Figure 4.3 showing a significant increase in the release of RANTES, MCP-1, IL-6, and KC from the TNF-α treated C2C12 myotubes compared with untreated control cells.

4.4.2.1 IL-6 release by skeletal muscle cells

The release of IL-6 from muscle in response to treatment TNF-α reinforces the role of IL-6 as a key myokine, released in response to inflammation. IL-6 is both pro- (Haddad et al., 2005) and anti-inflammatory (Xing et al., 1998). Exercise models report IL-6 release by muscle in tandem with elevations in anti-inflammatory cytokines such as IL-10 (Steensberg et al., 2003). However, no detectable levels of

IL-10 were measured in response to treatment of muscle cells with TNF-α, thus, we hypothesis that IL-6 released in response to TNF-α is forming part of a pro-inflammatory cascade. Elevated levels of IL-6 are associated with loss of skeletal muscle mass. Thus, infusion of IL-6 in rodents causing significant downregulation of ribosomal s6 kinase, which induces a catabolic state resulting in muscle atrophy (Haddad et al., 2005). Elevated circulating levels of IL-6 in circulation are an indicator of severity and predictor of mortality in sepsis (Hack et al., 1989, Damas et al., 1992). Moreover, inhibition of IL-6 using antibody blocking resulted in improved survival in a mouse model of sepsis (Riedemann et al., 2003). Therefore, our data suggest muscle can act as a significant source of circulating IL-6 in the critically ill, which may contribute to muscle atrophy and mortality in these patients.

4.4.2.2 MCP-1 release by skeletal muscle cells

The presence of highly chemotactic factors released from muscle, in response to TNF-α suggest a primary role as part of the early phase immune response, signalling to a variety of immune cells to instigate an inflammatory cascade. MCP-1 is primarily a monocyte chemoattractant, inducing the infiltration of circulating moncytes into tissues or sites of inflammation/infection. Monocytes and macrophages are a significant source of cytokines such as TNF-α and IL-1β which contribute to the overall hyperinflammatory state and the loss of muscle mass and function in the critically ill (Lang and Frost, 2007). Thus we hypothesise that myokines act as chemoattractants to recruit immune cells into muscle, which exacerbate the state of inflammation and contribute to the overall muscle atrophy and dysfunction.

Impaired insulin signalling is characteristic of the critically ill. MCP-1 has been associated with impaired insulin signalling and glucose uptake in skeletal muscle (Sell et al., 2006). Previously MCP-1 was thought to be derived from adipose tissue; however, data here suggest muscle may act as a source of MCP-1, which may have an autocrine signalling effect back onto skeletal muscle. Elevated levels of MCP-1 have been described to have deleterious effects (Ramnath et al., 2008) however, studies which knockdown MCP-1 expression have demonstrated impaired muscle repair and regeneration in response to injury (Shireman et al., 2007). Thus, MCP-1 clearly has a positive role to play in muscle regeneration, a process which is impaired in the critically ill. In contrast, elevated levels of MCP-1 in circulation of the critically ill are associated with poor clinical outcome. Investigation into therapies which suppress MCP-1 expression in sepsis show improved survival (Ramnath et al., 2008). Thus, the role of MCP-1 in the critically ill is poorly understood, however, the suggestion that muscle may act as a significant source implies it may contribute to the hyperinflammatory state and MOF.

4.4.2.3 RANTES release by skeletal muscle cells

RANTES is an eosinophil chemoattractant, its presence in and release by smooth muscle is well defined as having a major role in allergy associated asthma (Holgate et al., 1991). The presence of eosinophils (eosinophilia) in skeletal muscle is well defined in inflammatory myopathies, contributing to chemotaxis, in conditions such as polymyositis (Cantarini et al., 2009). Rodent models have reported RANTES expression is dependent on circulating TNF-α levels (VanOtteren et al., 1995). RANTES released from infiltrating macrophages disrupts muscle regeneration and

repair (Kohno et al., 2011) suggesting that muscle-derived RANTES may play a local role in the impaired muscle regeneration, which is evident in the critically ill.

4.4.2.4 KC release from skeletal muscle cells

KC is a potent neutrophil chemoattractant. The infiltration of neutrophils to muscle following contraction-induced damage is well defined, with a distinct role in tissue repair and remodelling (Zhang et al., 2009). During sepsis neutrophil infiltration and accumulation occurs in tissues and this is coupled with poor clearance of neutrophils (Brown et al., 2006). Such local production and release of KC and accumulation of neutrophils in muscle may contribute to the ROS-mediated dysfunction in muscle during systemic inflammation. Exacerbated production of superoxide (O_2^-) by neutrophils in response to the bacterial infection in sepsis is contributing factor towards morbidity (Alves-Filho et al., 2008). Furthermore, increased presence of nitric oxide (NO) results in the increased production of peroxynitrite ($ONOO^-$) a key player in the loss of muscle function in the critically ill (Callahan et al., 2001). Therefore, muscle is a significant chemotactic source to neutrophils and this is likely to contribute to the neutrophil-associated elevations in ROS present in muscle in the critically ill.

Thus, data indicate that in the presence of TNF-α, C2C12 myotubes activate and release a specific set of myokines as part of the inflammatory response. The profile of these cytokines centres around the early phase of the inflammatory cascade, recruiting monocytes, neutrophils and eosinophils in response to the cytokine stimulus and differs from the profile reported following exercise (Table 4.3). The accumulation of immune cells within muscle is widely described in several

inflammatory myopathies (Cantarini et al., 2009) and treatment of mice with TNF-α results in the accumulation of immune cells within skeletal muscle (Peterson et al., 2006). This infiltration was believed to be sustained and invoked by cytokine released from locally residing immune cells. However, data shown here suggest skeletal muscle can act as a cytokine reserve, playing a substantial role in the inflammatory response and has implications for the critically ill.. Data suggest that understanding the cross-talk between adipose tissue and skeletal muscle may be key to understanding and controlling inflammation in a number of conditions (Trayhurn et al., 2011). Thus, skeletal muscle secretes a similar array of cytokines compared to adipose tissue (Trayhurn et al., 2011)

4.4.2.5 Contribution of muscle-derived cytokines to systemic inflammation

The ability of muscle to release pro-inflammatory cytokines provides a new perspective to the role of muscle in inflammation. Skeletal muscle is the largest protein store in the human body and this raises the question as to the extent by which cytokines released by skeletal muscle contribute to the overall state of systemic inflammation. The highly chemotactic factors released from muscle in response to TNF-α have implications in a variety of pathologies. It can be hypothesised that the chemotactic factors are released to recruit immune cells to combat the invasive bacterial infection that underlies sepsis. Although required to combat the systemic infection, myokines may contribute significantly to the overall hyperinflammatory state in sepsis and local effects may contribute to the overall decline of muscle mass and function typically seen in sedentary critically ill patients. The attempt to use antagonists to TNF-α as a therapeutic agent in the critically ill has largely been unsuccessful (Fisher et al., 1996). This suggests perhaps that TNF-α is a key driver

of the inflammatory response and that its effect on muscle is important in the overall resolution of infection.

Myokine	Exercise	TNF-α treatment	Source
IL-6	+	+	mRNA Serum
IL-8	+		mRNA
IL-15	+		mRNA
IL-18	+		Protein
MCP-1		+	mRNA Protein
RANTES		+	mRNA Protein
KC		+	mRNA Protein
TNF-α	+		mRNA

Table 4.3 A summary of the myokines detected in and released from muscle in response to exercise or TNF-α treatment.

4.4.3 Examination of myokine release through golgi-mediated and exosomal pathways.

The release or exocytosis of factors from all cells is one that is well defined, highly stringent and pivotal for appropriate inter-cellular signalling. The endocytic pathway is a discrete mechanism whereby newly synthesized protein are post-translationally modified at the golgi and directed to the plasma membrane for exocytosis; where

membrane bound molecules are internalised and trafficking to the late endosome for degradation at the lysosome; or where proteins are trafficked from the golgi to the late endosome and through inward budding of its limiting membrane are internalised into nano-sized vesicles termed "exosomes" which then are released into the extracellular milieu. This non-classical pathway of exosome release permits release of proteins which lack leader sequences (Guescini et al., 2010) as well as mRNA and miRNA (Valadi et al., 2007). Furthermore, exosomes contain a variety of immunomodulatory agents (Admyre et al., 2007), such as cytokines, capable of modulating the immune response (Chen et al., 2011) and redox signalling, whereby exosomes caused elevations in ROS production, detected using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) a probe for general ROS production (Soderberg et al., 2007).

The release of exosomes from C2C12 myotubes raised the possibility that some myokines may use this process as a vehicle for exocytosis. Brefeldin A (BFA) blocks classical golgi-mediated transported using by intercalating between the cisternae of the golgi, inhibiting its trafficking capacity (Nebenfuhr et al., 2002). Non-classical release of exosomes can be ablated using a well characterised exosome blocker Dimethyl amilioride (DMA), which disrupts Na^+/H^+ Na^+/Ca^{2+} pumps in muscle due to the calcium dependent mechanism of exosome release (Savina et al., 2003). However, data from all four myokines studied demonstrated that no cytokines were released from skeletal muscle via the exosomal pathway in response to treatment with TNF-α. In contrast, all myokine release was dependent on classical golgi-mediated transport suggesting that exosomes are not the major route of myokines by skeletal muscle in this instance.

4.5 SUMMARY

It is widely believed that the source of cytokines at local sites of trauma/infection/inflammation/damage in muscle is infiltrating immune cells. Such cells do play a significant role in the inflammatory cascade; however, the capacity of muscle to act as an endogenous source of cytokines adds to the complexity. Based on the data presented here, the initial recruitment of immune cells to muscle is likely to be as result of local production of myokines.

Significant research in critical illness focuses on maintenance of muscle mass and function as a predictor of clinical outcome, a focus which is primarily based upoon the locomotive function of skeletal muscle. However, data shown here suggests that consideration must be given to the immunogenic capacity of skeletal muscle. Data presented here demonstrate the clear immunogenic role of muscle.

CHAPTER 5

EXPLORING THE CHAPEROKINE FUNCTION OF HSP60: ROLE IN EXTRACELLULAR SIGNALLING

5.1 INTRODUCTION

Data from Chapter 3 demonstrated that C2C12 myotubes release exosomes containing HSP60, following treatment with TNF-α. Extracellular HSP60 (eHSP60) has well defined immunomodulatory and signalling properties (Zhao et al., 2007, Cohen-Sfady et al., 2005), suggesting that muscle derived HSP60 may signal either in a paracrine manner on neighbouring muscle fibres or in an endocrine manner to more distal cells, organs and tissues. This chapter will examine the function of eHSP60.

5.1.1 Classical structure and function of HSPs

HSP60 is a nuclear encoded and mitochondrial localised member of the HSP family (Cheng et al., 1989). HSP60 forms a dimer structure in the mitochondrial matrix with HSP10 and this is known as the chaperonin complex. This chaperonin complex assists with folding of newly synthesised or imported proteins in the mitochondria in an ATP-dependent manner (Hartl, 1996). Proteins which are encoded in the nucleus of a cell and which contain a mitochondrial leader sequence are incorporated into the mitochondria via HSP75 in the matrix (Hartl, 1996). ATP facilitates the incorporation of newly synthesised proteins into the lumen of the HSP60/10 chaperonin complex to allowing correct conformational folding, with the production of ADP + Pi as products of the reaction.

In addition to the mitochondria, a population of HSP60 has also been located at the plasma membrane in cardiac muscle following cellular stress and where it is thought to be an indicator of cardiac failure (Lin et al., 2007).

5.1.2 The role of extracellular HSPs

HSPs were originally identified as intracellular proteins. However, recent research has indicated that HSPs also reside in extracellular environment. In this instance HSPs are termed eHSPs where it is proposed they may have different functions (Asea et al., 2000a). Thus, during instances of stress, in addition to the reported upregulation of HSPs in the intracellular environment (to promote cytoprotection) cells also release HSPs, notably HSP60 (Merendino et al., 2010, Gupta and Knowlton, 2007) and HSP70 (Wheeler et al., 2009, Giraldo et al., 2010b). Therefore, eHSPs have been termed "danger signals" that are capable of priming the immune system during the early stages of the inflammatory response. Thus, the term chaperokine has been widely used to describe eHSPs, as they have both chaperone and cytokine-like properties (Asea, 2006).

5.1.3 Immunomodulatory function of eHSPs

The first study to show the immunomodulatory function of eHSPs demonstrated that eHSP70 activated NFκB in human-derived monocytes and this resulted in the production of TNF-α, IL-1β and IL-6 by these cells (Asea et al., 2000b). eHSP72 also induces an inflammatory cytokine response in airway epithelial tissue, resulting in induction and release of IL-8 and TNF-α (Chase et al., 2007).

eHSP60 has been shown to interact with CD4+ CD25- regulatory T-cells, in a Toll-like receptor (TLR) 2 dependent manner (Zanin-Zhorov et al., 2006). eHSP60 upregulates IL-6 and IL-10 release from human B-cells via TLR-4 (Cohen-Sfady et al., 2005) and in adipocytes, to stimulate the release of IL-6, CXCL1 and MCP-1 (Gulden et al., 2008).

5.1.4 Role of eHSPs in disease states

Critically ill children with sepsis have elevated circulating levels of HSP60, with a direct relationship between serum HSP60 levels and severity of illness (Wheeler et al., 2007); whereby higher levels of eHSP60 are associated with morbidity in these patients. In patients with acute lung injury, elevated serum levels of HSP60 correlated with severity and progression of the respiratory trauma (Pespeni et al., 2005). Moreover, elevated circulating levels of HSP60 are correlated with cardiovascular disease risk (Shamaei-Tousi et al., 2007b). Thus, there is a clear association with elevated of eHSPs, particularly HSP60 in the circulation and a heightened inflammatory state.

Peripheral blood mononuclear cells (PBMCs) from patients with juvenile dermatomyositis show an exacerbated response to eHSP60 by up-regulation of IL1-β, IL-10 and TNF-α (Flohe et al., 2007). Studies examining neutrophil phagocytosis showed that exercise induced eHSP72 was capable of augmenting Peripheral blood mononuclear cells (PBMCs) from patients with juvenile dermatomyositis respond to eHSP60 by up-regulation o f IL1-β, IL-10 and TNF-α (Flohe et al., 2007). Studies examining neutrophil phagocytosis showed exercise induced eHSP72 was capable of augmenting the neutrophil response in a TLR-2 dependent manner (Giraldo et al., 2010a). Thus, there is evidence that eHSPs can modulate cytokine production by non-muscle cells.

5.1.5 Skeletal muscle-derived HSPs as extracellular signals

Skeletal muscle is the largest organ in the human body in terms of mass and protein and so this raises an important question; what contribution does HSP release by skeletal muscle make to the levels of HSPs exisiting in the circulation?

Extracellular HSPs clearly have an endocrine role to play, signalling to a whole variety of cells and tissues (De Maio, 2011). However, it is unknown whether endogenously synthesised HSP which are released from muscle then signal back in a paracrine manner; are synthesised and released from an alternative source to signal to skeletal muscle or signal to non-muscle cells and tissues.

Myokine	Treatment	Detected	Study
IL-6	Exercise	mRNA in human muscle biopsy. Protein in plasma.	Plomgaard et al. (2005) Pedersen & Fischer (2007)
IL-7	Differentiation	Primary human muscle cells.	Haugen et al. (2010)
IL-8	Exercise	mRNA in human muscle biopsy.	Akerstrom et al. (2005)
IL-15	Exercise Transfection	mRNA in muscle tissue. Protein in plasma	Pedersen et al. (2007) Quinn et al. (2009)
IL-18	Exercise	mRNA & protein in human muscle biopsy	Plomgaard et al. (2005)
TNF-α	Exercise	mRNA & protein in human muscle biopsy	Plomgaard et al. (2005)

Considerable evidence suggests that skeletal muscle is capable of releasing cytokines (termed myokines), particularly in response to exercise (Steensberg et al., 2002). The description of myokines raises the question to what extent does skeletal muscle contribute to the overall systemic inflammatory response in a number of stressful

situations, and whether extracellular HSPs play a role. Evidence shows the presence of eHSP receptors on the muscle plasma membrane, suggesting that this may be the case (Boyd et al., 2006).

5.1.6 Potential cross-talk between skeletal muscle and bone tissue

Throughout life bone tissue goes through a constant cycle of resorption and mineralisation, defined as bone turnover. Bone is replaced at a rate of 10% of the total skeleton per year (Parfitt, 1984). The process involves three bone cell-types, all with specific functions, as components of this cycle, namely osteoblasts, osteoclasts and osetocytes. Osteoblasts are involved in the formation of bone matrix and facilitate bone mineralisation (Croucher et al., 1994). SaOs-2 cells are well characterised osteoblast-like cell line, which retain the exact *in vivo* characteristics of bone metabolism, in terms of mineralisation (Rodan et al., 1987). Osteoclasts are involved in the removal of old bone tissue through resorption of mineralised bone (Teitelbaum, 2000). Osteocytes act as mechano-sensors responsible for the initiation of bone remodelling (Ehrlich and Lanyon, 2002). Typically it has been reported that bone-derived factors are the mediators of this response, however, exogenous agents such as IL-1, IL-6 and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) affect bone turnover (Compston 2001). An imbalance between bone removal by osteoclasts and bone formation by osteoblasts is the major causes of bone loss (Teitelbaum, 2000). Loss of bone results in weakness and frailty seen in disorders such as osteoporosis and in ageing (Croucher et al., 1994). The hyopthesis that skeletal muscle can release factors which may impact bone turnover suggests there may be a more local role for muscle derived signalling molecules.

Skeletal muscle not only provides locomotive force but also releases a number of signalling molecules. A greater understanding of the contribution to the immune system from skeletal muscle and its products is vital to understanding the subtle changes that occur both locally and systemically in both normal and pathophysiological states.

5.1.7 AIMS

The aims of this study were:

- To assess the stimulatory capabilities of HSP60 and exosomes in a paracrine manner upon C2C12 myotubes *in vitro* using purified HSP60 protein and exosome isolates.
- Determine the effect of HSP60 on the induction of the key inflammatory transcription factor NFκB in C2C12 myotubes
- Examine the effect of HSP60 on cytokine production by C2C12 myotubes
- Examine whether eHSP60 signalling to muscle cells occurs through TLR2/4.
- Determine the endocrine properties of HSP60 and muscle-derived exosomes through interaction with SaOs-2 osteoblast-like cells.

5.2 METHODS

5.2.1 The paracrine effect of HSP60 on C2C12 myotubes

5.2.1.1 Treatment of C2C12 myotubes with HSP60

Endotoxin contamination is an important consideration when carrying out stimulatory assays using commercial proteins (Osterloh et al., 2004). The presence of endotoxin in all treatment media was quantified using the Limulus Amoebocyte Lysate (LAL) assay, as described in Section 2.1.2.1.

Cellular viability was periodically assessed following all treatments, morphologically and using LIVE/DEAD viability staining described in Section 2.1.4 and caspase-3 protein content, using SDS-PAGE and western blotting as described in Section 2.3.

C2C12 myotubes were cultured *in vitro* using the protocol described in detail in Section 2.1.1. Cells were treated with 250, 500 or 1000ng/ml of endotoxin-free HSP60 for 3 hours (Zhao et al., 2007) to establish the optimum concentration of protein that induced the increased production of cytokines (Figure 4.1) as described in Section 2.1.2. The concentrations of HSP60 used are comparable to physiological levels (Shamaei-Tousi et al., 2006, Shamaei-Tousi et al., 2007b, Zhang et al., 2008). Optimal dose was determined by MCP-1 measurement in the cell culture media, using bioplex multi-bead analysis as described in Section 2.3.5.

In separate experiments, C2C12 myotubes were treated with HSP60 (1000ng/ml) and cytokine concentrations were determined in cell culture media at 1, 3, 6 and 24hrs following treatment, using Bioplex multi-bead analysis as described in Section

2.3.5. RNA was isolated from C2C12 cell lysates, and inflammatory gene expression was quantified using a PCR array, as described in Section 2.4.1.

Exosomes were isolated from C2C12 myotubes at 3 hours following treatment with TNF-α (25ng/ml), as described in Section 2.1.2. Total exosomal protein was quantified using the BCA assay as described in Section 2.3.1. C2C12 myotubes were treated with exosomes (50μg total exosome protein) for 1, 3, 6 and 24 hours. Cell culture media was harvested at these time points and subject to Bioplex multi-bead analysis for IL-6, KC, MCP-1 and RANTES as described in Section 2.3.4.

5.2.1.2 The effect of HSP60 on canonical NFκB activity in C2C12 myotubes

Nuclear fractions were isolated from control C2C12 myotubes and myotubes treated with TNF-α (25ng/ml) as described in Section 2.2.3. Localisation of the p65 subunit of transcription factor NFκB to the cell nucleus (as an indicator of activation of the canonical pathway of NFκB) was measured at the protein level using SDS-PAGE and western blotting, as described in Section 2.3.

5.2.1.3 Determination of the role of TLR-2/4 in eHSP signallling

To elucidate the precise mechanism of the action of HSP60 upon skeletal muscle, blocking studies were carried out by pre-treatment of C2C12 myotubes with antibodies to TLR-2 &-4, prior to HSP60 challenge as described in Section 2.1.1. Cytokine analysis and NFκB quantification was carried out as above, and described in Sections 2.3.4 and 2.3.

5.2.2 The effect of muscle derived signalling factors (HSP60 and exosomes) on bone mineralisation

5.2.2.1 Treatment of SaOs-2 osteoblast cells with HSP60 and muscle-derived exosomes

SaOs-2 Osteoblast-like cells (Rodan et al., 1987) were cultured as described in Section 2.1.1. Cells were treated for 3 hours in 6 well plates in a total volume of 2ml of culture media. Cells were treated with either HSP60 (1000ng/ml) or exosomes (containing 50μg total protein) isolated from C2C12 myotubes treated with TNF-α as described in Section 2.2.1. Additionally a set of SaOs-2 cells were also treated with 2ml conditioned media taken from C2C12 myotubes treated with TNF-α, to assess the impact of muscle derived milieu and its contents on bone cells.

5.2.2.2 Measurements of markers of bone turnover in SaOs-2 cells

The effect of HSP60 or exosomes on bone mineralisation was quantified by measurement of markers of mineralisation including alkaline phosphatase (ALP), osteocalcin (OC) and procollagen 1 n terminal peptide P1NP (Tobias et al., 2001). Quantification of ALP, OC and P1NP was carried out using High Performance Liquid Chromatography (HPLC) (Pratt et al., 1992) kindly undertaken by the Department of Clinical Chemistry at the University of Liverpool.

5.3 RESULTS

5.3.1 Determination of endotoxin levels in preparations of HSP60 and TNF-α used to treat cells.

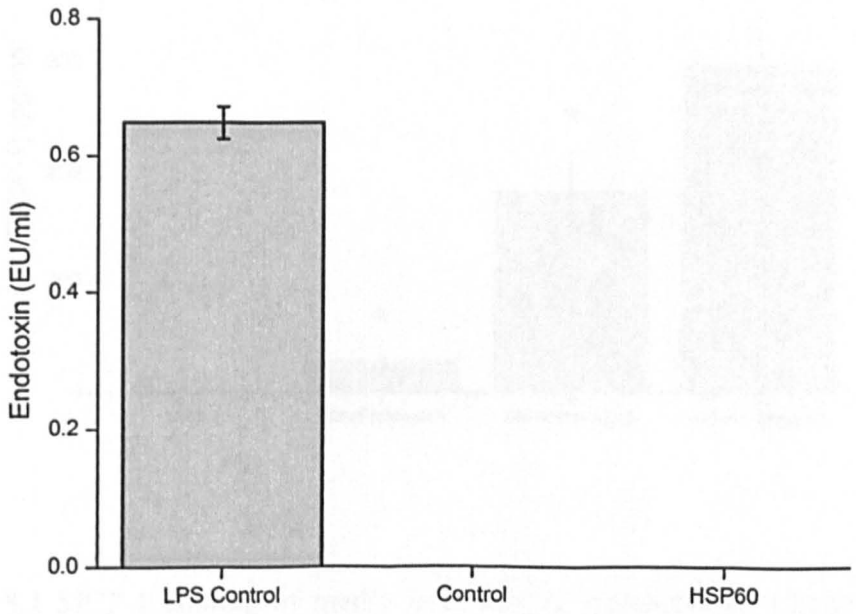


Figure 5.1 Endotoxin levels present in culture media of cells treated with LPS (1μg/ml) as a positive control, or HSP60 (1000ng/ml). Endotoxin levels were derived from a known *e.coli* standard. Data are presented as mean ± SEM (n=6; *p≤0.05).

No detectable levels of endotoxin were present in culture media preparations of HSP60 treated or the untreated control cells. Thus, the observed stimulatory capabilities of HSP60 were not as a result of contaminating endotoxin (Figure 5.1).

5.3.2 The effect of eHSP60 on cytokine release by C2C12 myotubes

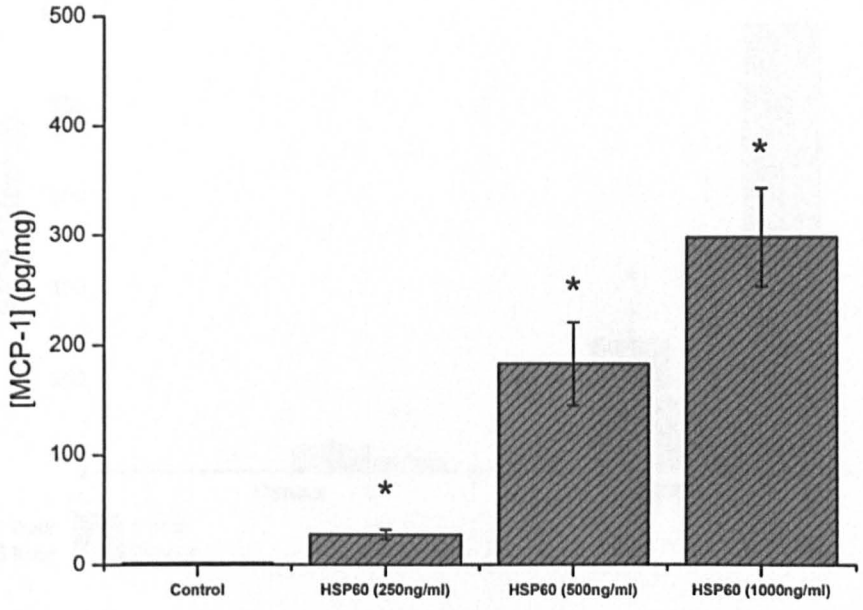


Figure 5.2 MCP-1 content of media from C2C12 myotubes at 3 hours following treatment with HSP60 (250, 500 or 1000ng/ml). Data are presented as mean ± SEM (n=4) *$p \leq 0.05$ compared with untreated control cells.

C2C12 myotubes were treated for 3 hours with recombinant endotoxin free HSP60 over a range of concentrations. A dose dependent release of MCP-1 was observed with a significant release of MCP-1 from HSP60 treated C2C12 myotubes compared with untreated control myotubes at all concentrations of HSP60 (Figure 5.2).

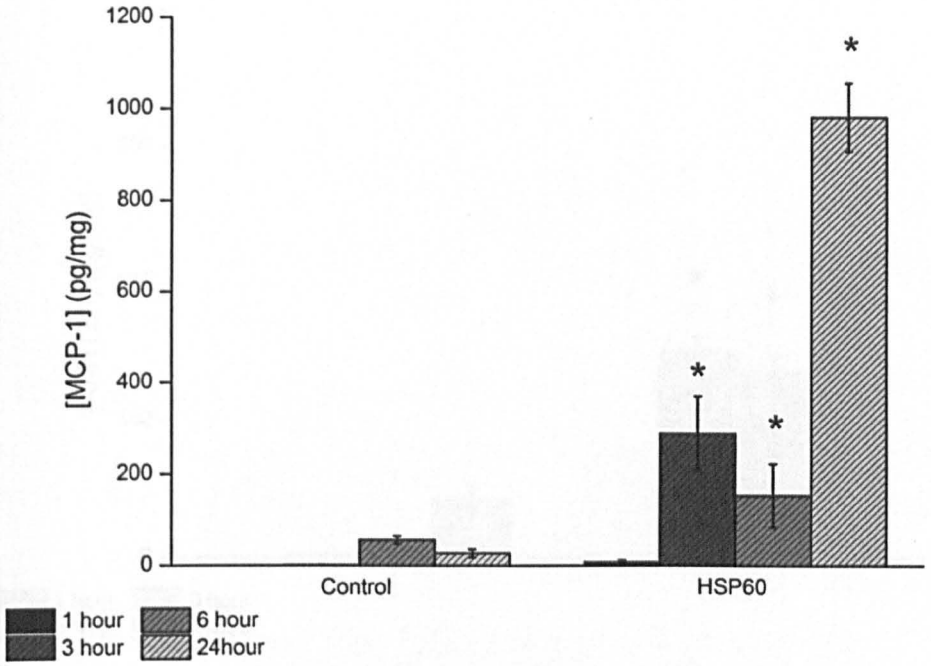


Figure 5.3 MCP-1 content of media taken from C2C12 myotubes following treatment with HSP60 (1000ng/ml), the dose used is representative of physiological levels occurring in circulation (Shamaei-Tousi et al., 2007). Data are standardised to total cellular protein and presented as mean ± SEM (n=6) *p≤0.05 compared with untreated control cells.

A significant release of MCP-1 by C2C12 myotubes was seen at 6 and 24 hours following treatment with HSP60 (Figure 5.3).

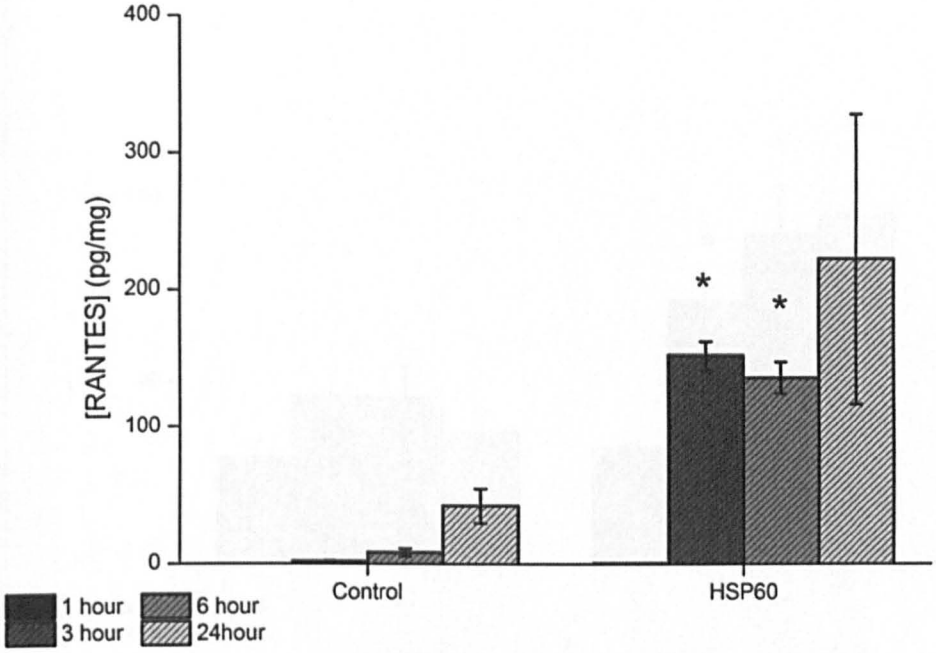


Figure 5.4 RANTES content of media from C2C12 myotubes following treatment with HSP60 (1000ng/ml). Data are standardised to total cellular protein and presented as mean ± SEM (n=6) *$p \leq 0.05$ compared with untreated control cells.

Significant release of RANTES by C2C12 myotubes was seen at 3 and 6 hours following treatment with HSP60. Mean levels of RANTES in the media were similar but rather variable at 24 hours following treatment with HSP60 (Figure 5.4).

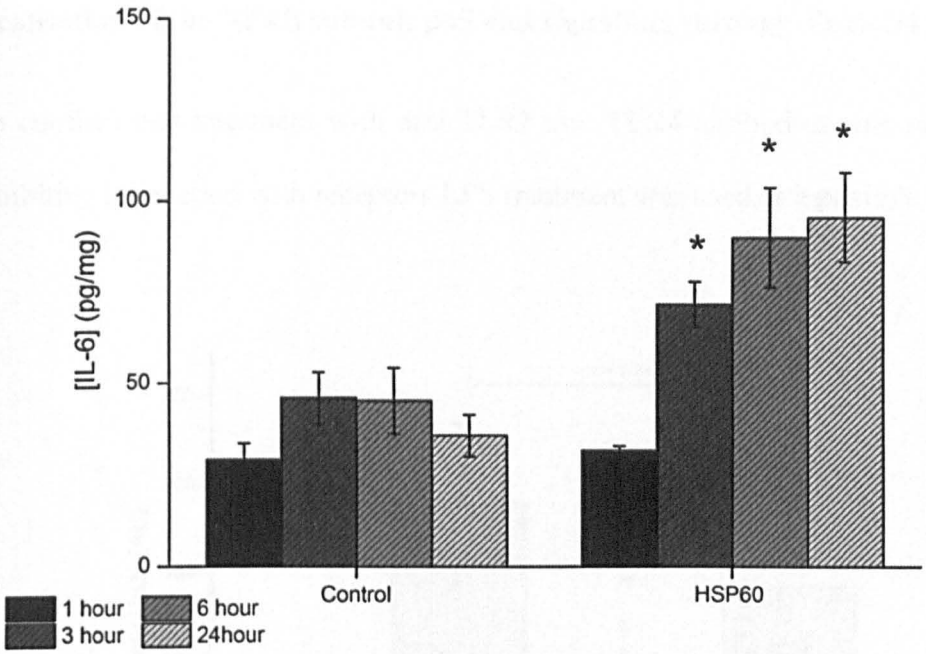


Figure 5.5 IL-6 content of media from C2C12 myotubes following treatment with HSP60 (1000ng/ml). Data are standardised to total cellular protein and presented as mean ± SEM (n=6) *p≤0.05 compared with untreated control cells.

C2C12 myotubes were treated over a time course with endotoxin free recombinant HSP60 (1000ng/ml). Significant release of IL-6 was observed at 3, 6 and 24 hours following treatment with HSP60, compared with the untreated control cells (Figure 5.5).

5.3.3 Determination of the effect of HSP60 and exosome treatment on nuclear localisation of the NFκB subunit p65 and signalling through TLR-2/4

To confirm that treatment with anti-TLR2 and TLR4 antibodies was successful in inhibiting interaction with receptors LPS treatment was used as a positive control.

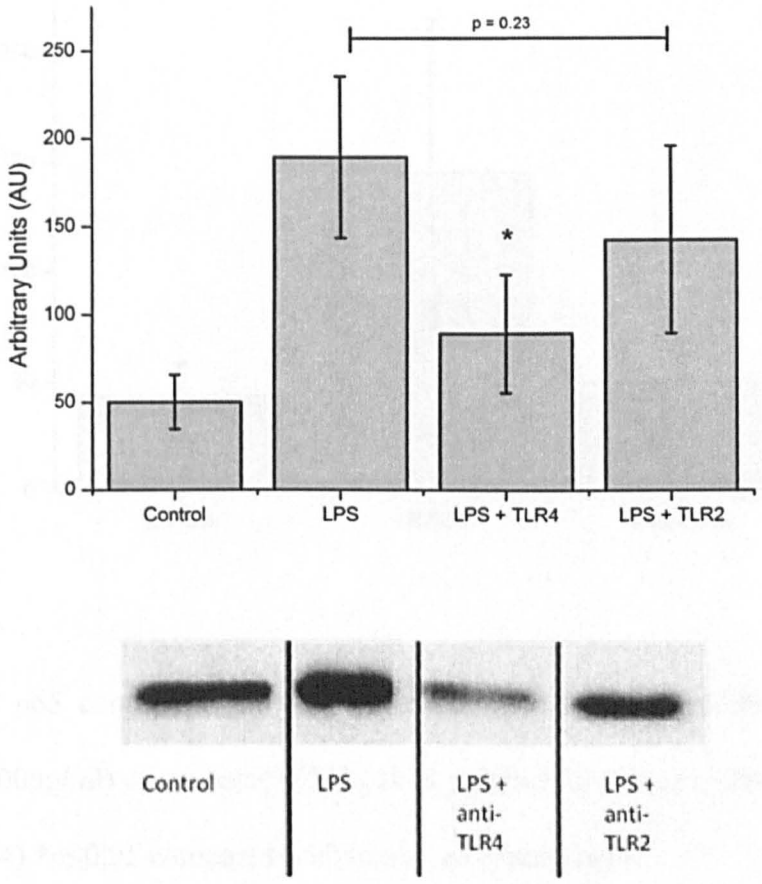


Figure 5.6 p65 content of the nuclear faction of C2C12 myotubes at 3 hours following treatment with LPS (1mg/ml) for 3 hours, with and without pre-treatment with anti-TLR2 or anti-TLR4 antibodies. Data are presented ± SEM (n=4) *p≤0.05 compared with untreated control cells.

Data show a significant increase in nuclear localised p65 folloing treatment with LPS. Pre-treatment with anti-TLR4 resulted in a significant reduction in nuclear p65

content. No effect of pre-treament with anti-TLR2 on p65 nuclear content was seen (Figure 5.6)

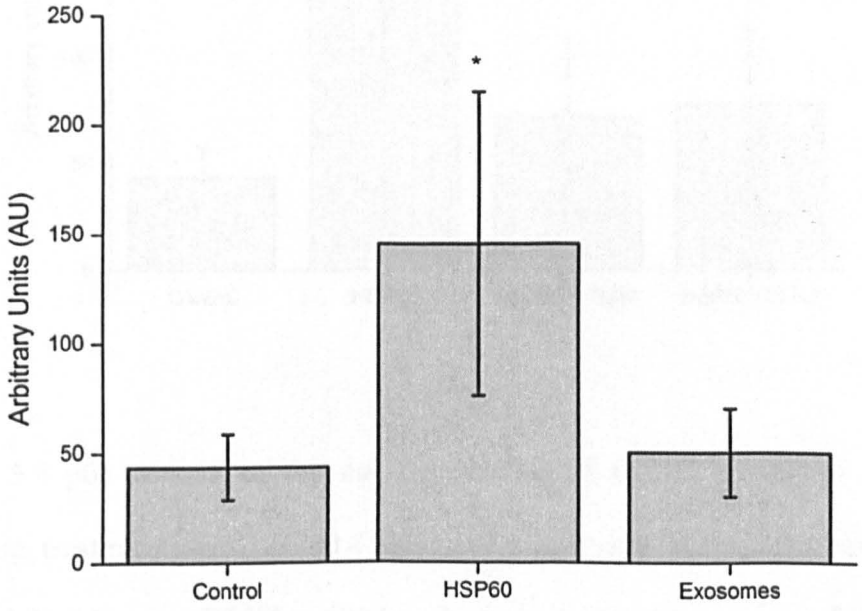


Figure 5.7 p65 content of the nuclear fraction of C2C12 myotubes treated with HSP60 (1000ng/ml) or exosomes (50µg total protein) for 3 hours. Data are presented ± SEM (n=4) *p≤0.05 compared with untreated control cells.

Data shows significant increase in nuclear localised p65 following treatment with HSP60, however, no significant change was observed following treatment with exosomes (Figure 5.7)

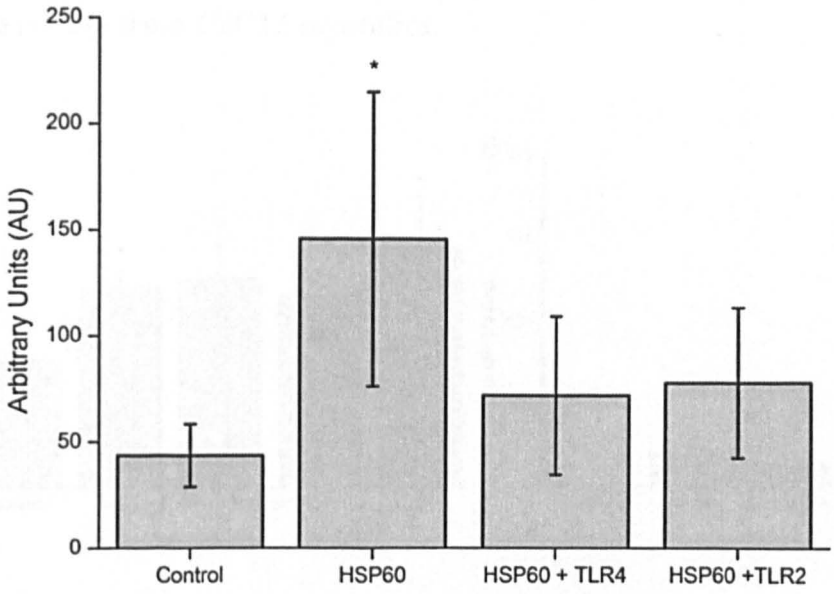


Figure 5.8 p65 content of the nuclear fraction of C2C12 myotubes at 3 hours following treatment with HSP60 (1000ng/ml) with and without the pre-treatment with anti-TLR2 or anti-TLR4 antibodies. Data are presented as mean ± SEM (n=4-8) *p≤0.05 compared with untreated control cells.

Significant elevation in the levels of nuclear localised p65 were observed in nuclei of myotubes treated with HSP60 compared with untreated cells; pre-treatment with anti-TLR2 and anti-TLR4 showed no significant reduction in nuclear localised p65 (Figure 5.8).

5.3.4 Determination on the effect of blocking TLR-2/4 on HSP60-induced cytokine release from C2C12 myotubes.

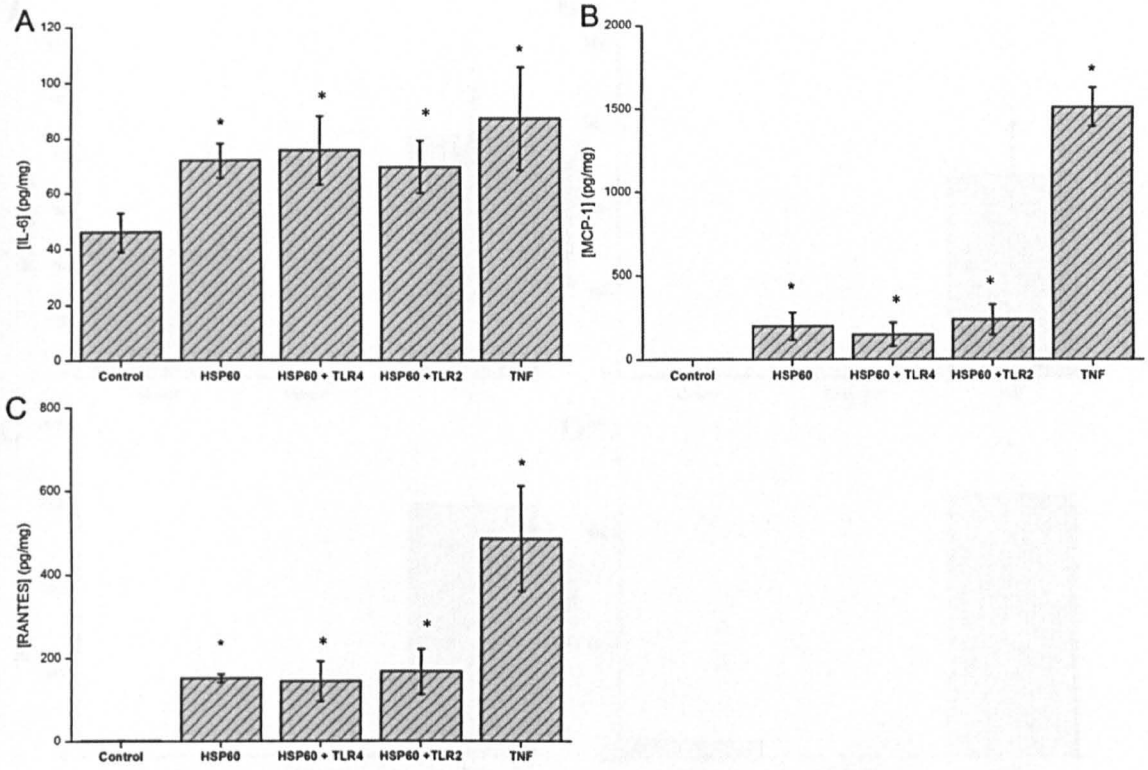


Figure 5.9 Levels of IL-6 **(A)**, MCP-1 **(B)** and RANTES **(C)** in media from C2C12 myotubes at 3 hours following treatment with HSP60 (1000ng/ml), with or without 1 hour pre-treatment with anti-TLR2/4. TNF-α (25ng/ml) was used as a positive control. Data are standardised to total cellular protein and presented as mean ±SEM (n=4) *p≤0.05 compared with untreated control cells.

Pre-treatment of cells with anti-TLR2 and anti-TLR4 had no significant effect on the levels of IL-6, MCP-1 and RANTES released from C2C12 myotubes following treatment with HSP60 (Figure 5.9). No detectable levels of KC were present in the cell culture media.

5.3.5 Determination of the effect of exosome treatment on cytokine release by C2C12 myotubes

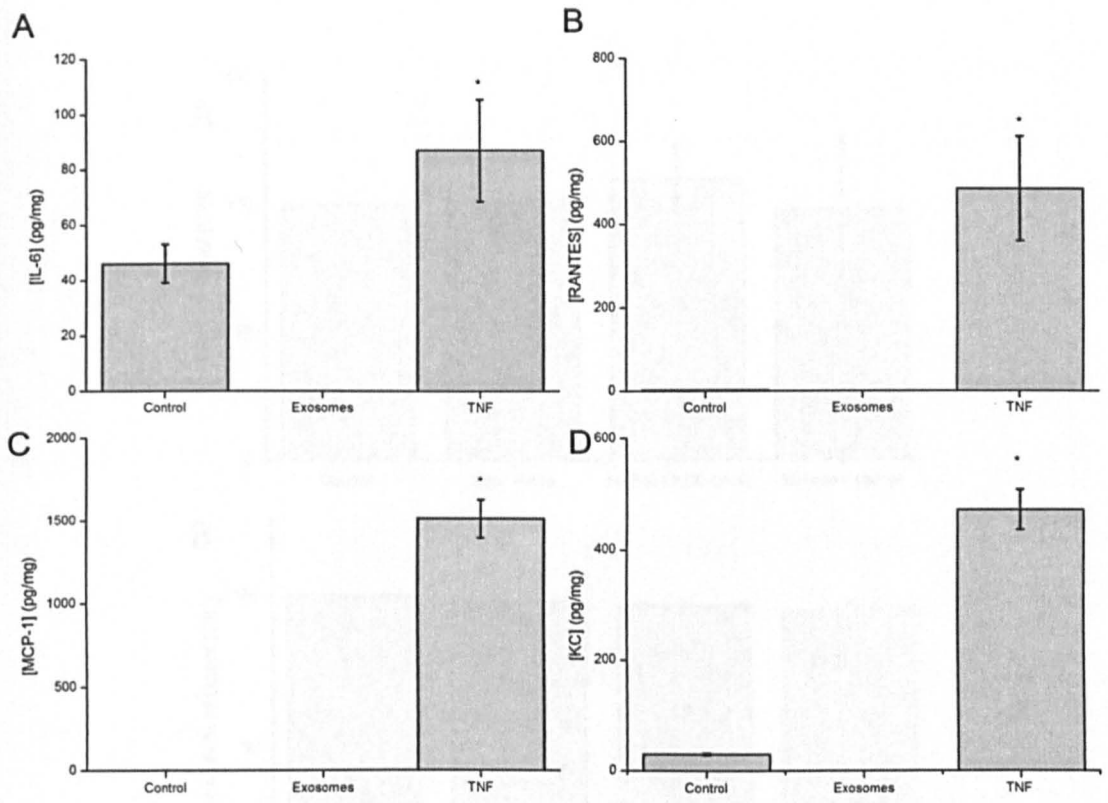


Figure 5.10 Levels of RANTES **(A)**, IL-6 **(B)**, KC **(C)** and MCP-1 **(D)** in media from C2C12 myotubes at 3 hours following treatment with exosomes (50μg of purified exosomes from C2C2 myotubes) or TNF-α (25ng/ml). Data are standardised to total cellular protein and presented as mean ±SEM (n=4) *p≤0.05 compared with untreated control cells.

No significant effect on cytokine content was observed in media when cells were treated with the exosome preparation. In contrast, significant increases in media content of cytokines was seen following treatment with TNF-α (Figure 5.10).

5.3.6 Determination of the effect of HSP60 and muscle-derived exosomes on SaOs-2 osteoblast-like cells.

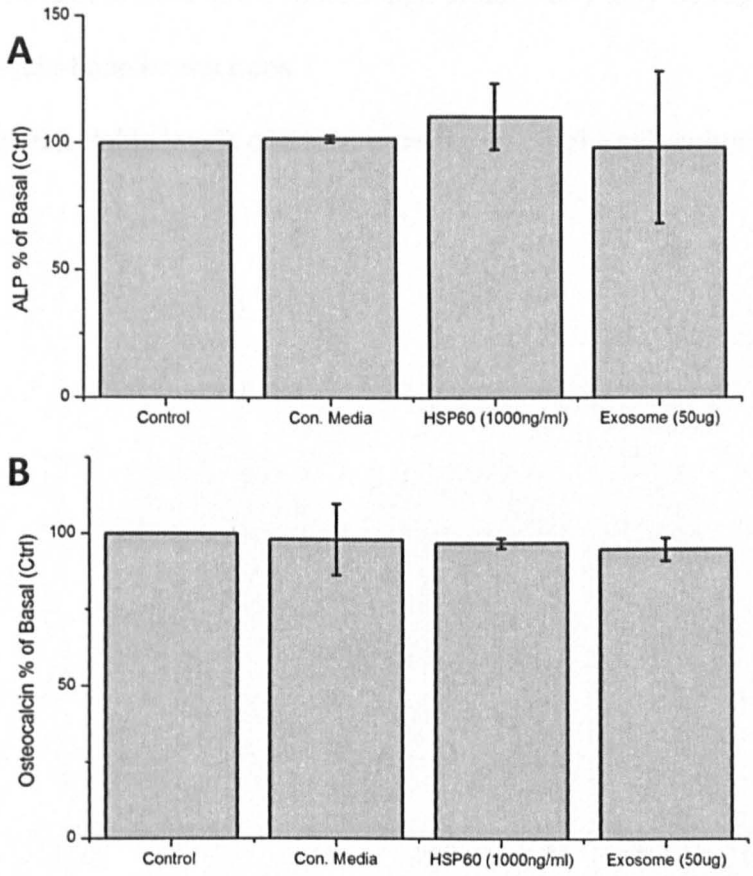


Figure 5.11 Levels of **(A)** Alkaline Phosphatase (AP) and **(B)** Osteocalcin (OC) measured in the culture media of SaOs-2 osteoblast cells treated for 3 hours with conditioned media (taken from TNF-α treated C2C12 myotubes), endotoxin-free recombinant HSP60 (1000ng/ml) or C2C12 myotube-derived exosomes (50μg total protein). Data are presented mean, as mean as % of the basal levels determined in media from untreated control cells ± SEM, (n=3-4)*$p \leq 0.05$ compared with untreated control cells.

No significant changes were observed in the levels of either AP or OC released from SaOs-2 cells in response to all conditions (Figure 5.11) indicating that muscle-derived factors have no significant effect on bone mineralisation over this time course. It is pertinent to note that a more longitudinal study may be required to fully understand muscle-bone interactions.

In addition, no detectable levels of P1NP were present in the cell culture media (data not shown).

5.3.7 Comparison of the levels of free HSP60 and HSP60 content of exosomes released from TNF-α treated C2C12 myotubes.

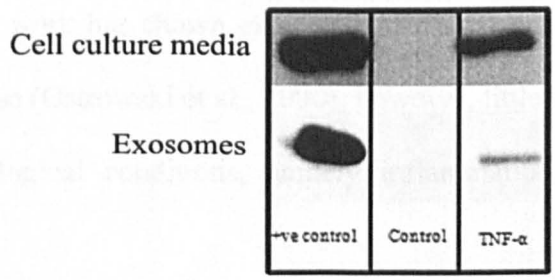


Figure 5.12 Representative western blot showing levels of HSP60 in the cell culture media and exosome fraction from control C2C12 myotubes or myotubes at 3 hours following treatment with TNF-α (25ng/ml) compared with a recombinant HSP60 positive control (2.5μg). Fifty micrograms of total protein was loaded for each of the control and TNF-α treated samples.

Based on the average intensity for each band data show approximately 40ng/50μg total protein, of HSP60 is released from C2C12 myotubes into the cell culture media. Of that 40ng approximately 67% (27ng) is located within exosomes (Figure 5.12).

5.4 DISCUSSION

Research pioneered by Dr Bente Pedersen and colleagues over the last decade has demonstrated that skeletal muscle is a significant source of cytokines (termed myokines). Previous work has shown exercise to be a primary inducer of myokine production and release (Ostrowski et al., 2000); however, little work has investigated the effect of pathological conditions, namely inflammation upon this cytokine response.

Work from Chapter 3 demonstrated that C2C12 myotubes release HSPs in response to treatment with TNF-α. This suggests that skeletal muscle can act as a source of eHSPs, an additional immune-modulator to myokines. Since skeletal muscle has the largest protein store in the human body it can be hypothesised that muscle can act as a significant source of endocrine signalling molecules such as cytokines and eHSPs.

Clearly skeletal muscle is a key source of endocrine signalling factors and the work undertaken in this Chapter aimed to elucidate the paracrine and endocrine signalling capabilities of eHSPs.

The aims of this study were:

- To assess the stimulatory capabilities of HSP60 in a paracrine manner upon C2C12 myotubes *in vitro* using purified HSP60 protein and exosome isolates.
- Determine the effect of HSP60 on the induction of the key inflammatory transcription factor Nuclear factor kappa B (NFκB) in C2C12 myotubes
- Examine the effect of HSP60 on cytokine production by C2C12 myotubes
- Examine whether eHSP60 signalling on muscle cells occurs through TLR2/4.
- Determine the endocrine properties of HSP60 through interaction with SaOs-2 osteoblast-like cells.

5.4.1 The effect of HSP60 on cytokine release from C2C12 myotubes.

An important aspect of any study using recombinant proteins (Kol et al., 2000) is to ensure that any myokine release was independent of endotoxin contaminants in the protein preparation. Preparations were analysed for the presence of endotoxin using the Limulus Amoebocyte Lysate (LAL). There were no detectable levels of endotoxin in the cell culture media examined from control untreated, and HSP60-treated preparations (Figure 5.1). These data indicate that the myokine release from C2C12 myotubes induced by either HSP60 or TNF-α treatment was not associated with contaminating endotoxin.

HSP60 is a key signalling molecule to T-cells, activating the discrete TLR- specific pathway and promoting cytokine up-regulation and production (Cohen-Sfady et al., 2005, Zanin-Zhorov et al., 2003). This study assessed the effect of eHSP60 upon cytokine production and release by C2C12 myotubes, initially determining the dose response of HSP60, on MCP-1 release from C2C12 myotubes. C2C12 myotubes

were treated for 3 hours with HSP60 (250, 500 or 1000ng/ml), MCP-1 levels showed a dose dependent response to HSP60 treatment compared with untreated control cells (Figure 5.2). These data are the first indication that HSP60 can act in an immunostimulatory and paracrine manner on skeletal muscle cells. From these data an optimal dose of 1000ng/ml was used in future analyses. The doses used of HSP60 are physiological levels occurring in circulation of healthy individuals (Shamaei-Tousi et al., 2007b). In a study of over 457, 40% of volunteers had levels of HSP60 $\geq$ 1000ng/ml in circulation. Data support the stimulatory capability of HSP60 in mononuclear cells and further investigation in human monocytes (Zhao et al., 2007) dendritic cells (Flohe et al., 2003) and cardiac muscle (Kim et al., 2009). Significant increased release of MCP-1, RANTES and IL-6 was seen between 3 & 24 hours following treatment of C2C12 myotubes with HSP60 (Figures 5.3-5.5).

These data show for the first time that eHSP60 has a function whereby it acts as a signalling molecule to skeletal muscle, to induce the production and release of cytokines. The cytokines released, particularly MCP-1 and RANTES are pro-inflammatory, suggesting skeletal muscle plays a role either locally and systemically in the early-phase immune response. Elevated levels of MCP-1 are found in the circulation of critically ill patients with sepsis (Bozza et al., 2007) and prevention of MCP-1 expression in rodent models of sepsis showed improved survival. Improved outcome was correlated with reduced levels of myeloperoxidase, an enzyme responsible for the production of ROS such as hydrogen peroxide (H_2O_2) and the hyperchlorous ion (HOCl). The reduction in myeloperoxidase was due to the suppressed neutrophil response due to the reduced MCP-1 expression (Ramnath et al., 2008). Thus, we hypothesise that muscle can act as a source of MCP-1 – which may contribute to the exacerbated neutrophil response during sepsis and subsequent

muscle dysfunction. Elevated levels of RANTES are also typically present in the critically ill. RANTES, primarily secreted from macrophages has a major impact on suppression of muscle regeneration in rodent models of endotoxin-induced inflammation (Kohno et al., 2011). Thus, data shown here suggests muscle may act as a source of RANTES, which may contribute to the impaired muscle regeneration seen in the critically ill.

The release of IL-6 from muscle cells in response to HSP60 is supportive of the previously described role of IL-6 as a key myokine (Keller et al., 2003b). Moreover, these data support studies indicating IL-6 expression responds to a variety of TLR ligands in muscle (Frost et al., 2006). However, it is unclear whether IL-6 induced by eHSP60 is acting in a pro- or anti-inflammatory manner. High circulating levels of IL-6 are associated with muscle wasting (Haddad et al., 2005), whereas subtle elevations in IL-6 are reported to be anti-inflammatory (Steensberg et al., 2003). The use of an *in vitro* cell system to study the effect of eHSP60 on skeletal muscle is important. However, we are unable to determine to what magnitude eHSP60-induced myokines contribute to the systemic inflammatory cascade.

The immunostimulatory capability of eHSP60 has been widely characterised (Cohen-Sfady et al., 2005) and taken together with data from Chapter 3, raises the hypothesis that skeletal muscle derived eHSP60 can signal in a paracrine manner to neighbouring muscle fibres or other cells, fitting the description of eHSPs as "danger-signals" whose role is to prime the immune response, whereby eHSP60 induces the release of myokines pivotal to the early stages of the inflammatory cascade.

5.4.2 Investigation of the signalling processes involved in HSP60-induced myokine release from C2C12 myotubes.

The function of eHSPs, specifically eHSP60 as an immunomodulator has been reported to occur via signalling through TLR 2 and 4 (Zanin-Zhorov et al., 2006, Zanin-Zhorov et al., 2003, Cohen-Sfady et al., 2005). The signalling cascades downstream of TLR's is well characterised with activation of NFκB, defined as a pleiotropic transcription factor. The ability of eHSP60 to activate the NFκB pathway is well defined (Chung et al., 2009). In addition, extracellular HSC70 has been shown to induce KC and MCP-1 expression via TLR-4 in cardiac muscle (Ao et al., 2009). Therefore it was hypothesised that eHSP60 could activate NFκB in C2C12 myotubes via activation of TLR-2/4.

5.4.3 The effect of eHSP60 on NFκB activation in C2C12 myotubes.

The effect of eHSP60 on NFκB activation was analysed by western blot analysis for subunit p65 in isolated nuclear fractions. NFκB is sequestered in the cytosol of cells in an inactive state bound to IκB. Cellular stress invokes the activation of NFκB by the degradation of IκB by Iκ Kinase (IκK) and the subsequent translocation to the nucleus to induce targeted gene transcription, including cytokines; this is termed the canonical pathway of activation (Hayden and Ghosh, 2011).

Addition of lipopolysaccharide (LPS) to C2C12 myotubes as a positive control induced a significant increase in p65 content of the nuclei of myotubes. TLR4 is the ligand for LPS and pre-treatment with antibodies to TLR-4 resulted in significant reduction in LPS-induced nuclear localisation of p65 (Figure 5.6). Pre-treatment with anti-TLR2 antibodies showed no significant change in LPS-induced nuclear localisation of p65 in C2C12 myotubes (Figure 5.6). These data confirm the efficacy

of the anti-TLR antibodies, particularly anti-TLR4, to specifically block the TLR successfully.

Data show that eHSP60 has a potent ability to activate NFκB in C2C12 myotubes in a similar manner to eHSC70 (Ao et al., 2009) this increased nuclear localisation of p65 was associated with increased myokine release (Figures 5.3-5.5) suggesting that the eHSP60-induced release of myokines occurs via activation of the canonical NFκB pathway. In addition, it may be that eHSP60 released from muscle may play a role in the persistent activation of NFκB in the critically ill, and thus contribute to the loss of muscle mass and function associated with NFκB activation.

To determine whether the increased nuclear content of p65 following treatment with eHSP60 occurs via the activation of TLR-2/4. C2C12 myotubes were pre-treated with antibodies to TLR-2/4, as described in Section 2.1.2. However, there was no significant reduction in nuclear p65 content when cells were pre-treated with antibodies to both TLR2 and TLR4 (Figure 5.7).

These data presented in Figure 5.7 do not confirm the widely described mechanism of eHSP activation of TLR-2/4. Although the expression of TLR2 and TLR4 is widely described in skeletal muscle (Kim et al., 2010) the ligands of eHSPs are not fully understood. The specificity of eHSPs for certain receptors needs to be considered carefully. By definition HSPs bind to a wide variety of proteins to stabilise folding and ensure correct tertiary structure. Thus, we hypothesise that eHSPs have an affinity for a large range of proteins/receptors in the extracellular environment. Therefore, it is likely that eHSPs can signal via a wide spectrum of receptors.

To examine this effect further the effect of blocking TLR2/4 on the release of HSP60 induced myokines was determined. Figure 5.8 shows levels of IL-6, MCP-1 and RANTES in media of C2C12 myotubes in response to treatment with eHSP60. Inhibition of TLR2/4 resulted in no significant change in myokine release induced by eHSP60. These data suggest that eHSP60-induced myokine release occurs independently of the toll-like receptor signalling pathway. There may be alternative eHSP receptors expressed on the surface of skeletal muscle which are involved in eHSP induced myokine release. Recent research has demonstrated that RAW cells respond to eHSP60 by the acitation of ADAM-7, vascular cellular adhesion molecule-1(VCAM-1) and immunoglobulin binding protein (BiP) receptors (Henderson and Mesher, 2007). Moreover, in CD^{4+} T-cells which express CD91, CD91 is a ligand for eHSPs (Fischer et al., 2010). Overall these data suggest eHSP60-induced activation of NFκB and subsequent increased production of myokines is TLR-independent.

5.4.4 C2C12 myotube derived exosomes and their paracrine signalling properties.

The study of the signalling properties of exosomes was problematic due to the low yield from muscle cells, making titration over a range of concentrations technically difficult.

Data demonstrated that incubation of C2C12 myotubes with intact exosomes, containing 50μg of exosomal protein resulted in no response in terms of myokine release or nuclear localisation of NFκB (Figure 5.7 and 5.10). Although these data suggest that exosomes purified from C2C12 myotubes (containing HSP60) have little effect on paracrine signalling to skeletal muscle, the exosomes may have a

greater affinity for other cells and tissues. Research into the targeting of cells/tissues with exosomes has demonstrated that specific peptide sequences expressed on the surface of exosomes affect the affinity of these proteins to the target cell (Alvarez-Erviti et al., 2011). However, the effect of exosomes on skeletal muscle may indeed be subtle. Exosomes are widely described as a means of genetic transfer (Zhou et al., 2011). Exosomes can be internalised within cells and can transfer mRNA and miRNA to target cells causing changes in gene expression and expression of novel proteins (Valadi et al., 2007) and this may be one of the role so muscle-derived exosomes.

Data presented in this chapter has demonstrated that when C2C12 myotubes are treated with levels of HSP60 which are physiological. The HSP60 is capable of activating NFκB and inducing cytokine release. Data in Chapter 3 demonstrated that exosomes are the main vehicle for the release of HSP60 from muscle in response to treatment with TNF-α. However, treatment of C2C12 with quantities of exosomes previously described to be capable of stimulating cyotkine production in non-muscle cells (Zitvogel et al., 1998), resulted in no detectable change in cytokine release. Therefore this raises an important question; what proportion of HSP60 released from muscle is exosome-associated and how does this compare to the levels of HSP60 reported in to be circulating *in vivo* in a number of conditions. Data shown in Figure 5.12 demonstrate that, of the 40ng of HSP60 released from C2C12 myotubes at 3 hours following treatment with TNF-α (from a total of 50μg of cellular protein) was associated with exosomes. Thus, 50μg of exosomes-derived protein from C2C12 myotubes contains approximately 27ng of HSP60 protein (Figure 5.12). Therefore, to be able to treat C2C12 myotubes with physiological levels of HSP60 from muscle-derived exosomes (~1000ng/ml) this would require a 40-fold increase,

relating to approximately 5mg of exosomal protein. Thus, it is unlikely that the level of HSP60 contained in exosomes is high enough to activate the release of cytokines from muscle. Due to the low yield and labour intensive methods of exosome isolation from cell culture achieving these levels would be technically very difficult. Therefore, the treatment with 50μg of exosomes bearing ~ 30ng of HSP60 would not be sufficient to stimulate cytokine release when we consider the dose response of HSP60 shown in Figure 5.2. Although levels occurring physiologically are around 1000ng/ml, the low amount released from muscle, in exosomes, suggests that muscle is not a significant contributor to HSP60 levels in circulation during systemic inflammation. We hypothesise that the majority of circulating HSP60 is derived from a non-muscle source. However, the impact of HSP60 in circulation in terms of cytokine up-regulation and release from muscle cannot be understated.

5.4.5 The effect of HSP60 and muscle derived exosomes on bone mineralisation in cell culture.

Bone mineralisation is the process whereby new bone tissue is formed by osteoblasts following resorption of old bone tissue by osteoclasts. This process is regulated by several key factors, namely osteocalcin (OC), alkaline phosphatase (AP) and Procollagen 1 n terminal peptide (P1NP). It is reported that AP derived from osteoblasts is an enzyme involved in mineralisation of the bone matrix, although the precise mechanisms of its role are poorly understood (Lumachi et al., 2009). Generally speaking AP is a well characterised marker of bone mineralisation and an indicator of turnover. Osteocalcin, secreted by osteoblasts, is also key marker of bone mineralisation, which is typically characterised by elevated serum levels of OC (Lumachi et al., 2009). Similarly, P1NP a member of the family of procollagen

peptides, is cleaved by the liver and incorporated into the bone matrix during formation (Watts, 1999). No detectable changes in AP, OC or measurable levels of P1NP were observed when osteoblast-like cells were treated with HSP60, intact exosomes (50μg exosomal protein) or conditioned media (Figure 5.11). Such findings were surprising since a variety of cytokines and hormone-like factors have been implicated in the augmentation of bone turnover (Datta et al., 2008). With respect to the lack of effect of conditioned media on SaOs-2 cells (media from C2C12 myotubes treated with TNF-α (25ng/ml for 3 hours) the concentrations of myokines present in the conditioned media could be below the threshold necessary to elicit changes in bone mineralisation. Osteoblasts express TLR4 upon their surface (Kikuchi et al., 2001); therefore it was surprising that HSP60 treatment using physiological doses had no effect on the release of markers of bone mineralisation. However, recent studies indicate alternative receptors to TLRs, such as ADAM-7, ICAM-1 and BiP, however, it is unclear whether muscle or bone ubiquitously express these receptors (Henderson and Mesher, 2007). Therefore, we conclude that the stimulatory capacity of HSP60 is not wholly dependent on the TLR signalling pathway. Recent research has indicated that HSP60 augments enhanced bone resportion in osteoclasts, in oestrogen deficient conditions (Koh et al., 2009). This suggests that augmentation of bone turnover may be dependent on the compounded effect of a number of signalling factors in the extracellular environment, rather than a sole "master regulator". Treatment of SaOs-2 cells with exosomes demonstrated no change in markers of bone mineralisation. However, the field of exosomes is in its relative infancy and the physiological concentrations of exosomes in circulation are poorly understood. Moreover, recent research has indicated exosomes can be targeted to specific tissues (Alvarez-Erviti et al., 2011) however it unclear to what

extent this occurs naturally during exosome biogenesis. This suggests that exosomes may need to be pre-targeted to a tissue to be able to elicit any stimulatory effect. However, exosomes derived from skeletal muscle are poorly characterised. Thus, data suggest that skeletal muscle-derived exosomes may not possess any ability to modify bone turnover.

5.5 SUMMARY

The data in this chapter shows the capability of eHSP60 to signal to skeletal muscle and induce the release of myokines. These data support the "chaperokine" function of heat shock proteins, whereby they perform chaperone duties in the intracellular environment and have cytokine-like properties in the extracellular environment.

The significant release of MCP-1 and RANTES in response to eHSP60 infers that skeletal muscle may be involved in the early-phase immune response, either through local interactions or to more distal organs and tissues. The release of IL-6 in response to HSP60 is intriguing, since IL-6 has been described to be both pro- and anti-inflammatory (Pedersen et al., 2003). The role of IL-6 could be as a primer of an anti-inflammatory response, eliciting up-regulation of factors such as IL-1ra and IL-10 (Steensberg et al., 2003).

The release of both pro- and anti-inflammatory cytokines by muscle as a result of HSP60 treatment or exercise suggests they may play a role in the overall system of resolution, repair and remodelling seen in instances of trauma (Tidball, 2005). Alternatively, the role of myokines may be more systemic. Exercise studies have shown elevation in plasma levels of cytokines (IL-6) believed to be derived from muscle. However, there is no evidence published to demonstrate the effect of chronic inflammation on myokine release *in vivo*.

Although the field of eHSPs is in its infancy, there are numerous studies that have defined specific signalling pathways involved in the biological role of eHSPs. eHSPs have been shown to bind TLRs and induce inflammatory response in a variety of cells and tissues (Ao et al., 2009, Chase et al., 2007). These data show however that HSP60 induced myokine production and NFκB up-regulation is not dependent on TLRs as demonstrated in previous studies. However, recent research has indicated that eHSPs may interact via scavenger receptors such as CD91. The hypothesis that there is broad range of eHSP receptors is one that is gathering significant attention.

The finding that intact C2C12 myotube derived exosomes do not invoke an immunostimulatory function in a paracrine manner contradicts the immuostimulatory function of exosomes in part. However, the skeletal muscle derived exosomes may act on other cells and tissues – for instance, locally, to neuromuscular junctions. Exosomes can be targeted to target specific cells/tissue based up expression of specific peptide sequences upon the targets outer membrane (Alvarez-Erviti et al., 2011).

Overall, these data confirm the immunostimulatory function of eHSP60, however, it unclear of the specific signalling pathways involved. Furthermore, HSP60 bearing exosomes derived from skeletal muscle may have endocrine signalling properties to distal cells and tissues. However, this precise function remains unclear.

CHAPTER 6

THE EFFECT OF TNF-α TREATMENT ON HSP PRODUCTION AND MYOKINE RELEASE FROM SKELETAL MUSCLE *IN VIVO*

6.1 Introduction

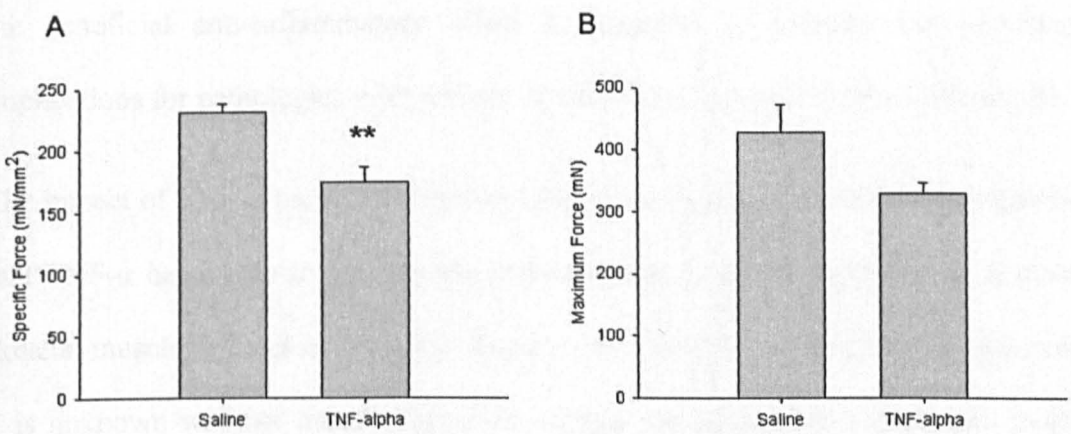
6.1.1 Elevated circulating levels of TNF-α *in vivo*: impact on muscle mass and function in the critically ill patient.

Muscle weakness, in particular of the diaphragm, in the critically ill is profound and is exemplified by the difficulty in weaning patients from mechanical ventilation (Griffiths and Hall, 2010). Loss of force produced by skeletal muscle has been correlated with a reduction in fibre cross-sectional area in the critically ill, occurring at a rate of 4% per day (Helliwell et al., 1998) and a loss of muscle mass of 2% per day. The mechanisms by which this functional deficit occurs are poorly understood, although there is considerable evidence that elevated circulating levels of cytokines, particularly TNF-α have a major detrimental effect on muscle function. Elevated levels of TNF-α results in the increased production of reactive oxygen species (ROS) by muscle, which is also associated with loss of muscle through a number of mechanisms including the initiation of apoptosis (Supinski and Callahan, 2006). The sedentary nature of the critically ill also plays a significant role in the pathology of sepsis. Physical activity is a pivotal component of maintaining whole-body homeostasis. Inactivity induces remodelling of skeletal muscle, with a shift in fibre-type from type I to type II, a typical occurrence in the critically ill (Stein and Wade, 2005). The viscous cycle of TNF-α driven inflammation, muscle wasting, weakness and the associated physical inactivity are key determinants of morbidity in the critically ill (Griffiths, 1996).

There is a clear correlation between elevated circulating levels of TNF-α and poor clinical outcome in a variety of pathologies (Degens, 2010). In particular, the critically ill with sepsis exhibit severe systemic inflammation, and TNF-α is a key

component of that inflammatory cascade (Casey et al., 1993). A key effect of exposure of muscle to elevated TNF-α is cachexia (Saini et al., 2006). Although TNF-α is not the only pro-inflammatory cytokine in the sepsis cascade, it has a clear impact on skeletal muscle mass and function. Elevated circulating levels of TNF-α result in impaired force generation by skeletal muscle. Loss of force is associated with muscle weakness, typified by disruption of Ca^{2+} signalling in the myofilaments (Reid et al., 2002). Skeletal muscle weakness has been correlated with suppressed Ca^{2+} release from the sarcoplasmic reticulum (Westerblad et al., 2010). Muscle weakness in response to TNF-α treatment has been demonstrated in mice, whereby force generation was also depressed in the absence of muscle atrophy (Hardin et al., 2008). Data from our laboratory has further reinforced the impact of elevated circulating levels of TNF-α on muscle function in mice. Treatment of adult mice with 7.5μg/kg TNF causes a signifincant loss in specific force.

Figure 1.



Maximum specific force **(A)** and maximum tetanic force **(B)** of EDL muscles stimulated indirectly from saline control and TNF-α treated mice. Data are presented as mean ± SEM, n=5. *P<0.05 compared with control saline treated mice (Routely et al. in preparation)

6.1.2 Endocrine properties of skeletal muscle *in vivo*

The data presented in previous chapters has demonstrated the endocrine function of skeletal muscle *in vitro,* in agreement with the work Pedersen et al. e.g. (Febbraio and Pedersen, 2005). Contracting skeletal muscle releases cytokines into the circulation in both rodents (Jonsdottir et al., 2000) and humans (Keller et al., 2003b) independent of muscle damage. The majority of work examining skeletal muscle as an endocrine organ has focused on the release of cytokines by muscle following exercise. Exercise induces elevations in circulating IL-6 and this is accompanied by elevations in anti-inflammatory cytokines IL-10 and IL-1ra (Steensberg et al., 2003, Ostrowski et al., 2000, Pedersen and Febbraio, 2008) independently of any systemic elevation in TNF-α. Overall, this has formed the paradigm of exercise as a means of inducing a beneficial anti-inflammatory response *in vivo* (Pedersen et al., 2003). Studies have shown i.v administration of IL-6 during endotoxin insult is beneficial, ablating endotoxin induced elevation in TNF-α *in vivo* (Starkie et al., 2003). Overall the beneficial anti-inflammatory effect in response to exercise has important implications for pathologies with periods of inactivity, in particular the critically ill.

The impact of TNF-α on skeletal muscle catabolism is profound and we hypothesise that TNF-α has a role to play on the immunogenic function of muscle. It is clear, skeletal muscle releases cytokines in response to elevated levels of TNF-α, however, it is unknown whether muscle-derived cytokines are significant contributors to the systemic inflammatory cascade and/or have functions more locally.

6.1.3 Ageing and inflammation: impact on skeletal muscle

The loss of skeletal muscle mass and function in the critically ill is further complicated in older patients. By the age of 70, individuals have lost 30-40% of muscle mass, and the additional loss of (2%) per day can be catastrophic to the older patient. As we age there is a significant and aberrant elevation in circulating levels of pro-inflammatory cytokines (Visser et al., 2002). Low-grade systemic inflammation in the elderly is reported to be contributory to a variety of disorders. Moreover, elevated circulating levels of TNF-α have been demonstrated as a key controller of the inflammatory state of ageing (Roubenoff et al., 2003), with significant contribution to the loss of skeletal muscle in the elderly (Greiwe et al., 2001). There is substantial evidence that the environment of muscle in old individuals is pro-inflammatory (Conboy and Rando, 2005) but any compounded effect of systemic inflammation on the muscle of older individuals is poorly understood. Taken together with the release of muscle-derived cytokines we can hypothesise that muscle contributes to muscle dysfunction in both ageing and the critically ill through the release of myokines.

Based on work in previous Chapters it is clear that muscle is a minor source of HSPs *in vivo*, but a major source of cytokines. The role of cytokine production in muscle is therefore the primary focus of this Chapter.

6.1.4 Hypothesis

We hypothesise that:

1. Skeletal muscle adapts following elevation in circulating levels of TNF-α by an increased content of HSPs and cytokines.
2. Specific HSPs and cytokines are released by skeletal muscle *in vivo* which can impact on the whole body inflammatory response.
3. An inability to modulate the HSP content of muscles of old mice impacts on the production of cytokines by muscle and this contributes to the overall systemic inflammation in circulation of old mice.

6.2 Aims

The aims of the study were to:

- Investigate the effect of TNF-α treatment on HSP content of the *gastrocnemius* muscle and the serum of adult wild-type mice.
- To determine the effect of TNF-α treatment on the production and release of cytokines by the *gastrocnemius* muscle and serum of adult wild-type mice.
- To determine the effect of TNF-α treatment on the release of eHSPs and cytokines from isolated single muscle fibres of the *flexor digitorum brevis* (FDB) muscle from WT adult mice.
- To determine the effect of ageing on the ability of skeletal muscle to respond to TNF-α *in vivo* by changes in muscle HSP content and cytokine production.

6.3 Methods

6.3.1 Investigation into the effect of TNF-α treatment *in vivo* on HSPs in muscle and serum of WT C57Bl6 adult mice.

Adult mice (10-13 months) were randomly selected from a colony of C57Bl6 WT mice. The C57Bl6 strain is a well characterised murine species, of which the muscle physiology is well defined. C67Bl6 mice have analogous muscle deposition, function and ageing characteristics to that of humans.

Animals were treated with 7.5μg/kg of TNF-α in 200μl sterile saline or with saline as a control via the tail vein as describe in Section 2.5.1.1. Following treatment with TNF-α mice were culled at 1, 3, 6 and 24 hours following treatment. Muscle tissue and blood was removed and prepared as described in Section 2.5.2. Total muscle protein was quantified using the BCA assay as described in Section 2.3.1. HSPs were detected in muscle and serum by western blotting as described in Section 2.3.

6.3.2 Investigation of the effect of treatment of mice with TNF-α on cytokine concentrations in muscle and serum.

Following treatment of mice with TNF-α or saline for 1, 3, 6 and 24 hours mice were culled. Muscle tissue and blood was excised and prepared as described in Section 2.5.2. Total muscle protein was quantified using the BCA assay as described in Section 2.3.1. Cytokines were detected in muscle and serum using Bioplex multi-bead analysis as described in Section 2.3.5.

6.3.3 Investigation into the effect of treatment of mice with TNF-α on HSP and cytokine release from isolated single muscle fibres from WT C57Bl6.

Single muscle fibres from the *flexor digitorum brevis* (FDB) muscle of adult (10-13 months) and old (22-24 months) mice were removed as described in Section 2.5.4. Isolated single fibres were treated under cell culture conditions with TNF-α (25ng/ml). At 3 hours following treatment, cell culture media was analysed for the presence cytokines using Bioplex multi-bead analysis as described in Section 2.3.5 and analysed for the presence of eHSPs by ELISA as described in Section 2.3.4 Total protein in the isolated muscle fibres was quantified using the BCA assay as described in Section 2.3.1.

6.4 Results

6.4.1 The effect of TNF-α treatment on HSP content of *gastrocnemius* muscle of mice.

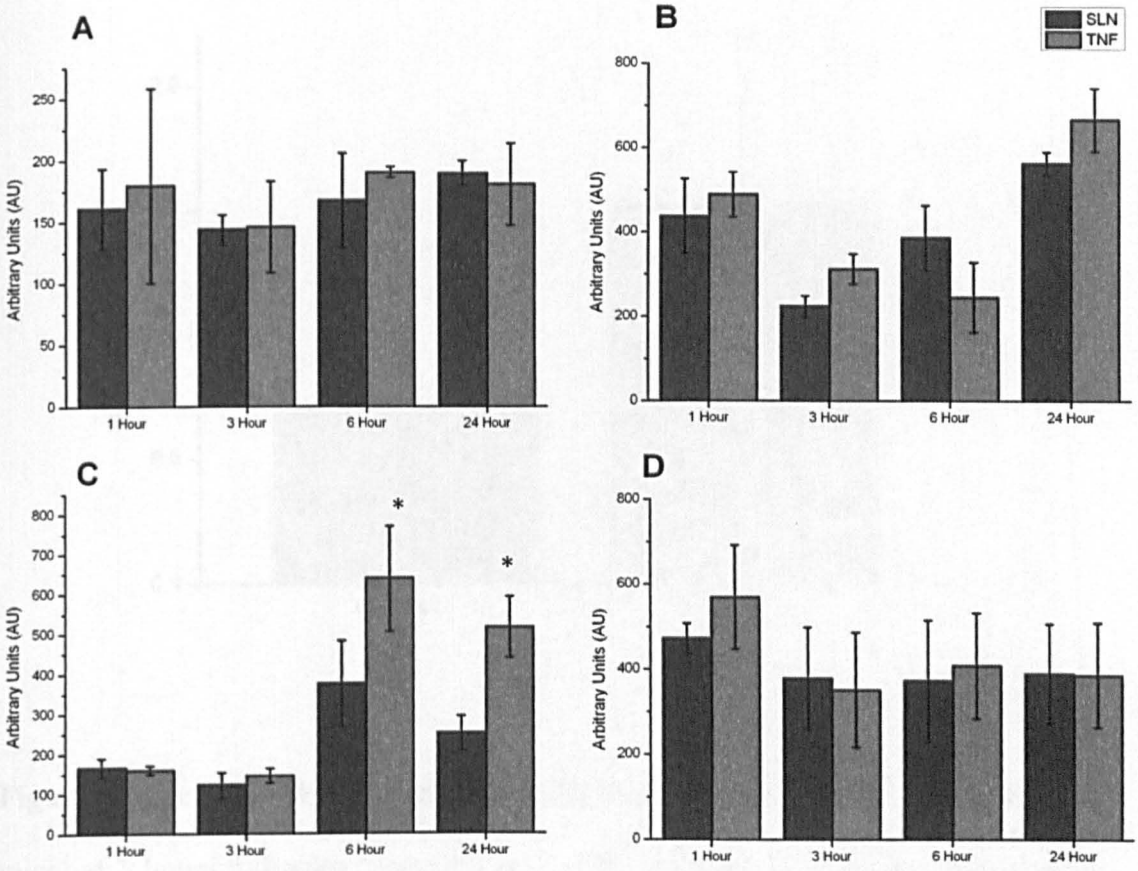


Figure 6.1 (A) HSP10, **(B)** HSP60, **(C)** HSP70 and **(D)** HSC70 content of the *gastrocnemius* muscle of adult WT mice at 1, 3, 6 and 24 hours following treatment with saline or TNF-α (7.5μg/kg) for. Data are presented as mean ± SEM (n=5) *p≤ 0.05 compared with saline treated control mice.

HSPs were readily detected in the *gastrocnemius* muscle of both saline and TNF-α treated adult mice. Significant increase in HSP70 content of the *gastrocnemius* muscle was seen at 6 and 24 hours following TNF-α treatment. However, no significant effects of TNF-α treatment were observed in HSP10, HSP60 and HSC70 at any time point (Figure 6.1).

6.4.2 The effect of TNF-α treatment on the release of HSPs from isolated single muscle fibres of adult WT mice.

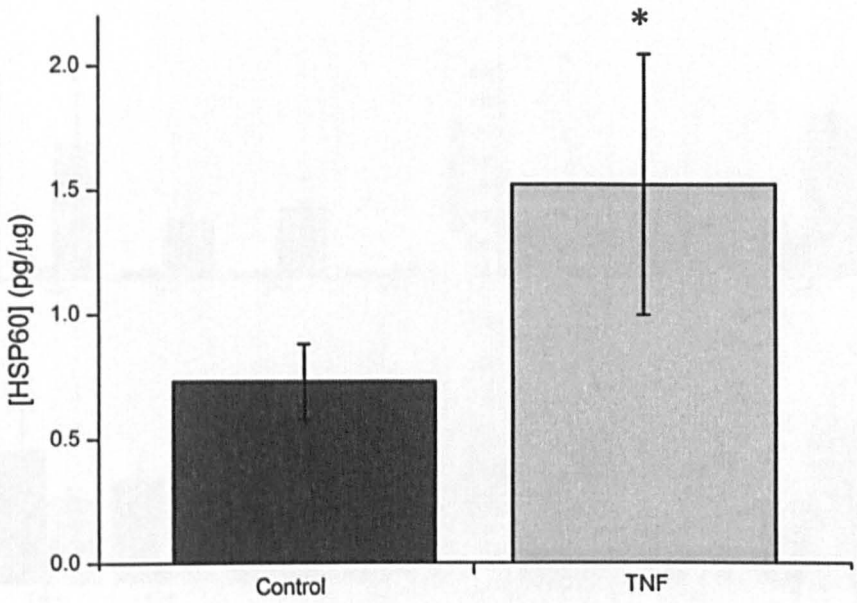


Figure 6.2 Levels of HSP60 released from isolated single muscle fibres of adult mice, at 3 hours following treatment with TNF-α (25ng/ml). Data are presented as mean ± SEM (n=5).*p≤0.05 compared with untreated control fibres.

HSP60 was readily detected in media from both control and TNF-α treated fibres; no detectable levels of HSP70 were evident. Significant elevation in HSP60 release from muscle was observed in response to treatment with TNF-α (Figure 6.2).

6.4.3 The effect of treatment of mice with TNF-α on cytokine levels in adult mouse *gastrocnemius* muscle.

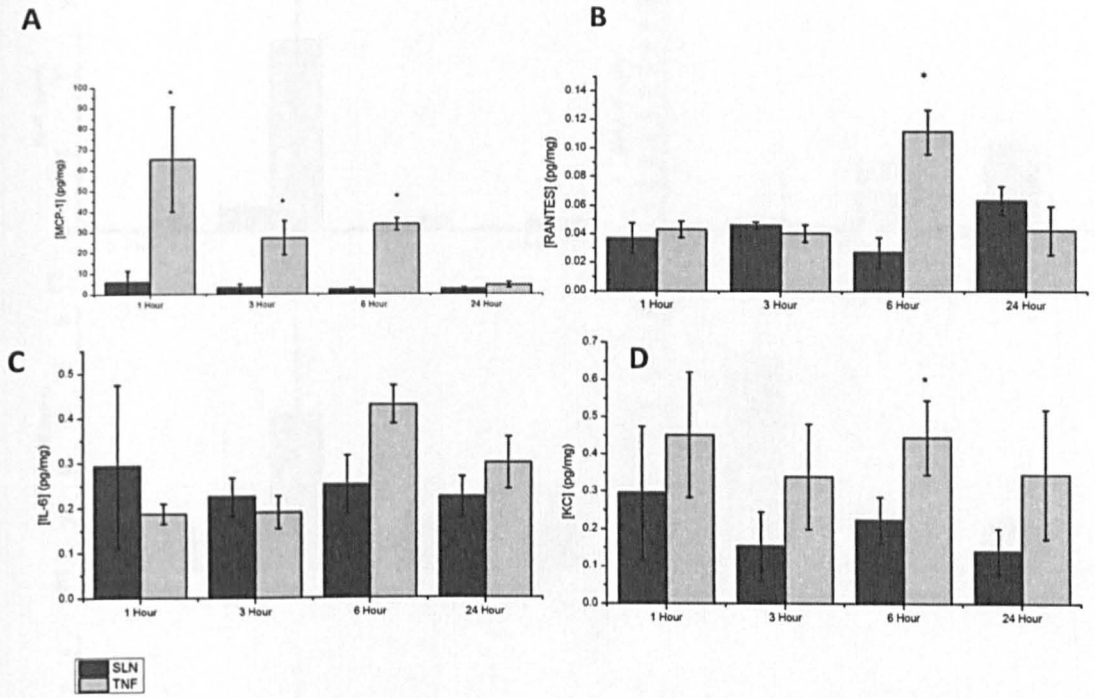


Figure 6.3 Levels of **(A)** MCP-1, **(B)** RANTES, **(C)** IL-6 and **(D)** KC in the *gastrocnemius* muscle of adult mice at 1, 3, 6 and 24 hours following treatment with saline (SLN) or TNF-α (7.5μg/kg). Data are presented as mean ± SEM (n=4) *p≤0.05 compared with saline treated control mice.

A significant increase of MCP-1 was detected in muscle following 1, 3 and 6 hours TNF-α treatment, compared with muscle of saline treated control mice; MCP-1 returned to basal at 24 hours. A significant increase in RANTES was observed at 6 hours following TNF-α compared with muscle of saline control mice. No significant change in concentration of IL-6 was seen following treatment with TNF-α when compared with saline control muscles. Significant rise in levels of KC was observed at 6 hours following treatment with TNF-α, compared with saline treated control muscles (Figure 6.3).

6.4.4 The effect of systemic administration of TNF-α on circulating serum cytokine levels.

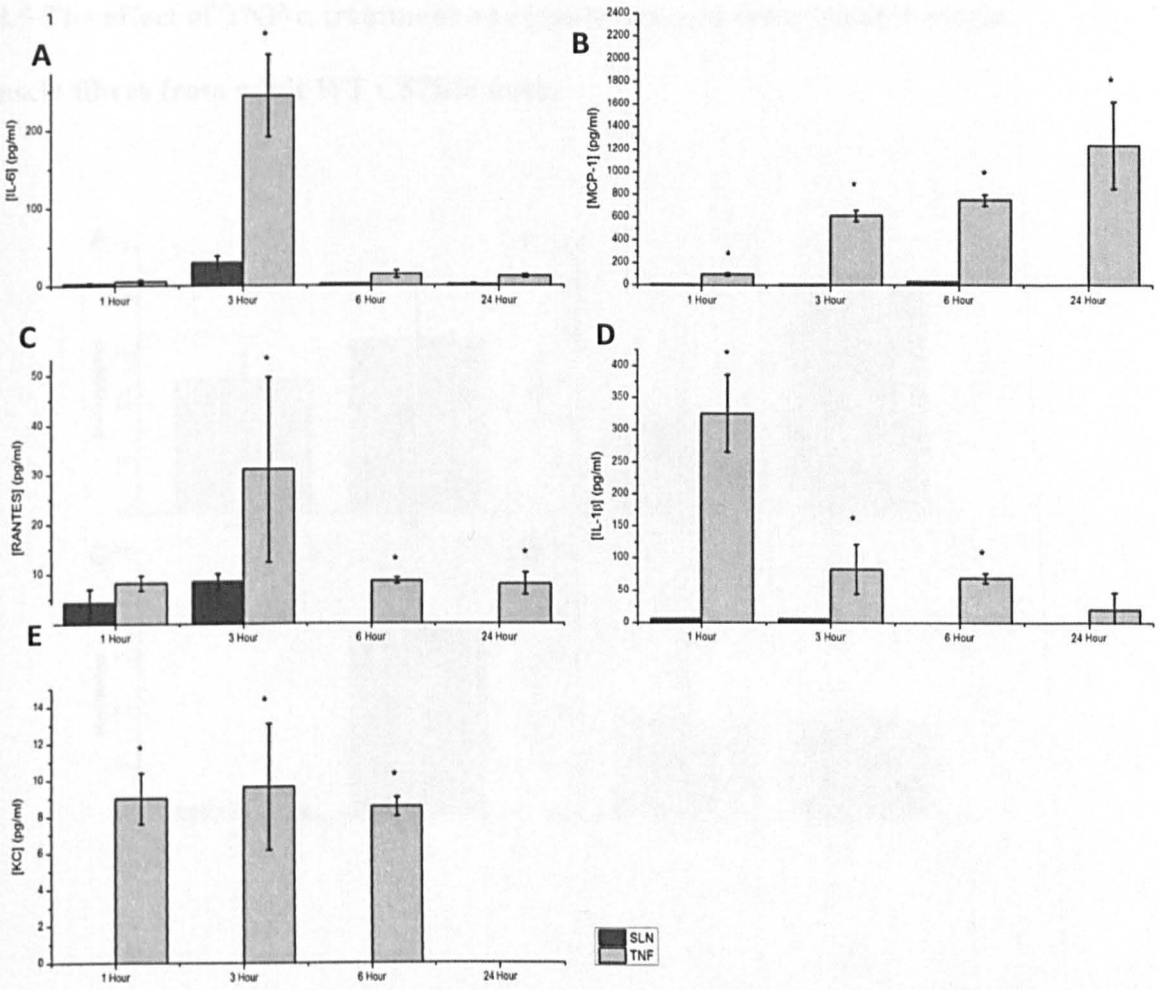


Figure 6.4 Levels of **(A)** IL-6, **(B)** MCP-1, **(C)** RANTES, **(D)** IL-1β and **(E)** KC in the serum of saline or TNF-α (7.5μg/kg) treated adult mice. Data are presented as mean ± SEM (n=4), *p≤0.05 compared with saline treated control mice.

A significant increase in IL-6 was observed in the serum of mice treated with TNF-α for 3 hours compared with saline treated control mice; serum levels returned to basal concentrations at 6 and 24 hours. A significant increase in MCP-1, RANTES, **(D)**

IL-1β and KC was detected in the serum of adult mice treated with TNF-α (Figure 6.4).

6.4.5 The effect of TNF-α treatment on cytokine release from isolated single muscle fibres from adult WT C57Bl6 mice.

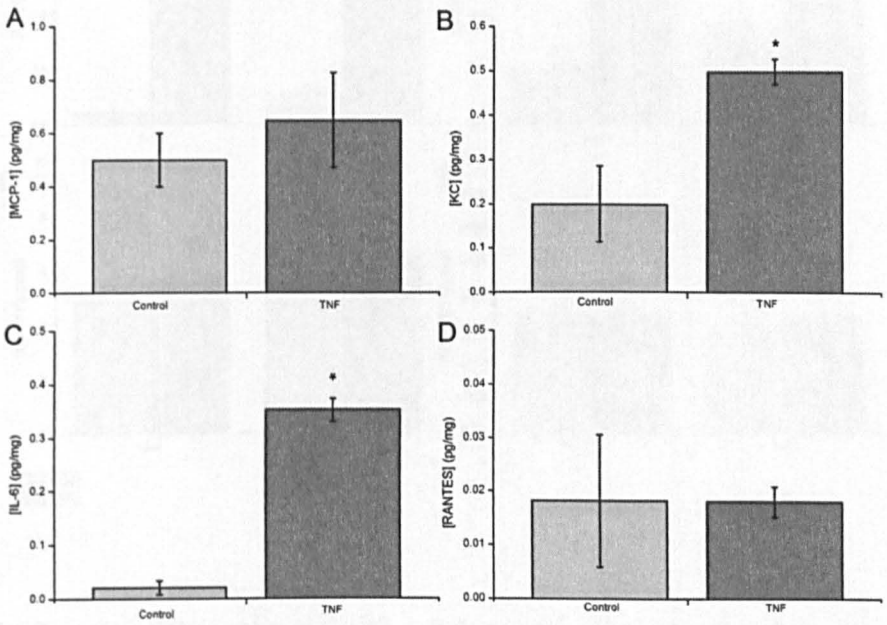


Figure 6.5 Levels of MCP-1 **(A)**, KC **(B)**, IL-6 **(C)** and RANTES **(D)** released from isolated single muscle fibres of adult mice at 3 hours following treatment with TNF-α (25ng/ml) compared with control untreated fibres. Data are presented as mean ± SEM (n=6).*p≤0.05.

Significant release of IL-6 and KC was seen from isolated muscle fibres following treatment with TNF-α. In contrast, no change was observed in MCP-1 and RANTES in following treatment with TNF-α (Figure 6.5).

6.4.6 Effect of TNF-α treatment on cytokine release from isolated single muscle fibres of adult and old mice.

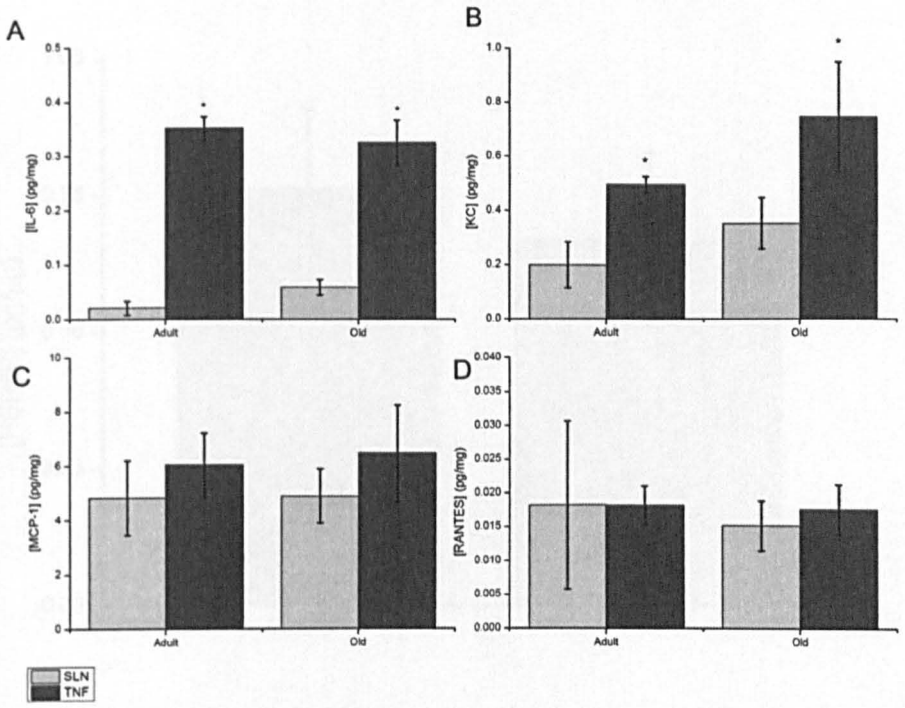


Figure 6.6 Levels of IL-6 **(A)**, KC **(B)**, MCP-1 **(C)** and RANTES **(D)** released from old (22-24 months) isolated single fibres from the FDB muscle in response to TNF-α (25ng/ml) treatment for 3 hours, compared with control untreated fibres. Data are presented as mean ± SEM (n=6).*$p \leq 0.05$. Adult data are taken from Figure 6.5, to allow comparison between data for adult and old.

Significant release of IL-6 and KC was seen from isolated single fibres in response to treatment with TNF-α. Detectable levels of MCP-1 and RANTES were present with comparable levels of old compared with young however; there was no effect of TNF-α treatment (Figure 6.6)

6.4.7 The impact of TNF-α on the release of HSPs from isolated single muscle fibres of old mice.

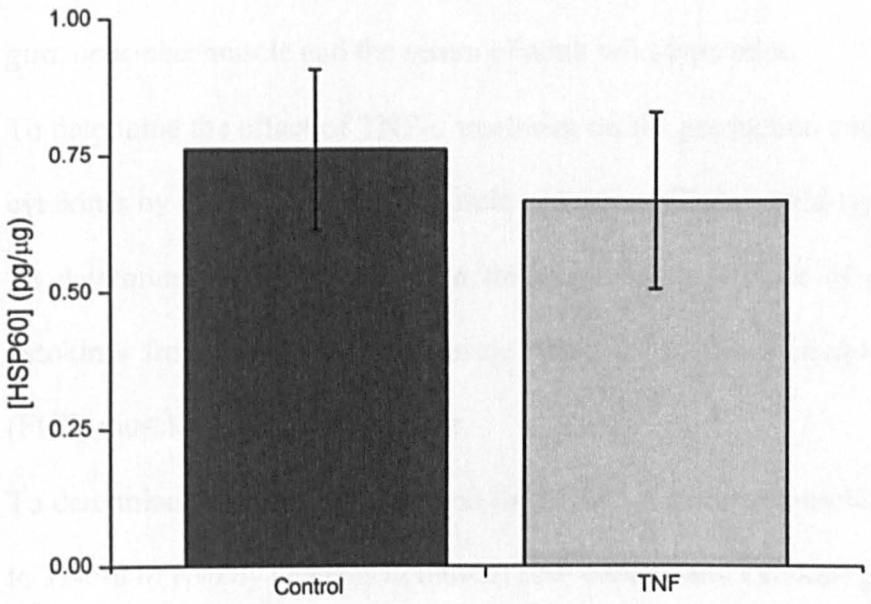


Figure 6.7 Levels of HSP60 released in media isolated single muscle fibres of old mice, following 3 hours treatment with TNF-α (25ng/ml). Data are presented as mean ± SEM (n=5).*p≤0.05.

HSP60 was readily detected in media from both control and TNF-α treated fibres of old mice; no detectable levels of HSP70 were present. No effect of treatment with TNF-α was observed in HSP60 release (Figure 6.8).

6.5 Discussion

The aims of the study were to:

- Investigate the effect of TNF-α treatment on HSP content of the *gastrocnemius* muscle and the serum of adult wild-type mice.
- To determine the effect of TNF-α treatment on the production and release of cytokines by the *gastrocnemius* muscle and serum of adult wild-type mice.
- To determine the effect of TNF-α treatment on the release of eHSPs and cytokines from isolated single muscle fibres of the *flexor digitorum brevis* (FDB) muscle from WT adult mice.
- To determine the effect of ageing on the ability of skeletal muscle to respond to TNF-α *in vivo* by changes in muscle HSP content and cytokine production.

6.5.1 The effect of TNF-α treatment on HSPs in the *gastrocnemius* muscle, serum and isolated fibres of adult wild-type (WT) C57Bl6 mice.

Data in Chapter 3 demonstrated that the treatment of C2C12 myotubes *in vitro* with TNF-α resulted in an increase in HSP content of muscle cells and a release of HSP60 into the extracellular environment. This study aimed to determine whether such mechanisms occur *in vivo* in response to TNF-α.

The up-regulation of HSPs within muscle as a means of cytoprotection is well described in human models of exercise and training (Morton et al., 2006, Morton et al., 2008) and murine models of both damaging (McArdle et al., 2004) and non-damaging muscle contractions (Broome et al., 2006). Data shown in Figure 6.1 demonstrates no significant change in muscle content of HSP10, HSP60 and HSC70

in response to TNF-α treatment, compared with saline treated control mice across the timecourse up to 24 hours. However, a significant elevation in the muscle HSP70 content was seen at 6 and 24 hours following TNF-α treatment. The significant increase in HSP70 is indicative of the widely characterised role of HSP70 as a highly sensitive stress inducible member of the HSP family (McArdle and Jackson, 1996). The lack of response of HSP10 and HSP60 is striking, since the *in vitro* model described in Chapter 3 showed up-regulation of both HSPs in response to TNF-α. In addition, the lack of response by HSC70 to TNF-α is also indicative of its cellular characteristic, being generally the least responsive member of the HSP family (Fink, 1999).

A small number of studies have demonstrated the presence of HSPs in circulation *in vivo*, in a number of conditions. Thus, investigation into septic children described elevated levels of HSP60 in circulation compared with healthy controls (Wheeler et al., 2007). Moreover, circulating HSP60 levels have been correlated with cardiovascular disease associated with diabetes (Shamaei-Tousi et al., 2007b, Shamaei-Tousi et al., 2006). The presence of HSP70 in circulation has been observed during pregnancy (Molvarec et al., 2010) and as marker of severity in plasma and sputum of asthmatic individuals (Changchun et al., 2011). Although the observation of circulating HSPs in a variety of (patho-) physiological states is one of great interest the precise function of extracellular HSPs (eHSPs) is poorly understood. Moreover, the source of circulating HSPs under such conditions is fundamentally unknown. We demonstrated previously in Chapter 3 that TNF-α induces the release of HSP60 from muscle *in vitro*, however the contribution to the overall circulating HSP60 pool is likely to be minor. Examination of serum taken from TNF-α and saline treated adult mice by ELISA showed no detectable levels of

HSP60 or HSP70 present in circulation, in both control and TNF-α treated mice. The majority of studies into circulating HSPs have been carried out in humans with only a small number of studies measuring HSPs in serum of mice, measuring HSP27 (Rayner et al., 2009) and HSP70 (Cai et al., 2010).

Levels of HSPs detected in these studies were in the low nanogram range, almost 1000 times less than levels reported in the human studies (Shamaei-Tousi et al., 2006, Shamaei-Tousi et al., 2007b). Based on these studies and work carried out in our laboratory we believe that there is currently not a sensitive enough ELISA assay to accurately quantify HSPs in murine circulation. In addition, the presence of albumin causes added problems. Albumin is the largest component of serum, and HSPs readily bind most proteins, thus we hypothesise that HSPs bind tightly to albumin and so this may result in an under estimation of the serum content of HSPs. Data shown in Figure 6.2 demonstrate that muscle releases HSP60 in response to TNF-α; these data support our findings in C2C12 myotubes described in Chapter 3. Given that fibres treated with TNF-α release approximately (1.51pg/μg ± 0.52) and the average muscle weight an adult mouse is 20g, comprising approximately 2g muscle weight we calculate that muscle would contribute to approximately 30ng of HSP60 in circulation, which is around 30% of levels reported in circulation. Thus, it is unlikely that muscle-derived HSP60 plays a significant role in inducing cytokine release from muscle and therefore exacerbating the hyperinflammatory state in the critically ill.

6.5.2 The effect of systemic administration of TNF-α on cytokine content of mouse *gastrocnemius* muscle.

The effect of inflammation on the cytokine response from muscle *in vivo* is currently unknown. Moreover, the contributing effect of muscle derived cytokines to the overall inflammatory cascade is also poorly understood. This study examined the effect of inflammation on the content of cytokines in muscle. Data shown in Chapter 4 demonstrated that TNF-α induced cytokine expression in C2C12 myotubes. Based on this the same cytokines were examined in whole *gastrocnemius* muscle from adult mice treated with saline or TNF-α *in vivo*.

Data shown are in general agreement with that seen *in vitro* (Chapter 4). Levels of MCP-1, RANTES, IL-6 and KC protein were readily detectable in the gastrocnemius muscle of mice as shown in Figure 6.3. TNF-α treatment induced significant elevation in MCP-1 content at 1, 3 and 6 hours following treatment and these levels returned to basal at 24 hours following treatment. Significant elevation in muscle RANTES and KC protein content was observed at 6 hours following treatment compared with saline treated control mice as shown in Figure 6.3. The changes observed at 1, 3, and 24 hours in RANTES and KC in response to TNF-α, may be due to the differential rates of translation of these proteins. Alternatively, elevated levels of RANTES and KC may have been released directly from the muscle into the circulation, hence, the whole muscle content remained unchanged at 1, 3 and 24 hours.

No significant change was observed in muscle IL-6 content between saline control and TNF-α treated mice (Figure 6.3). The function of IL-6 *in vivo* is controversial, with numerous studies implicating IL-6 role in both pro- and anti-inflammatory

processes. The classical sense, IL-6 was always proposed to be pro-inflammatory, and release from skeletal muscle during exercise, independent of TNF-α suggests it may be anti-inflammatory too. The phenomenon observed here in this suggest that TNF-α is driving a pro-inflammatory response (by elevating MCP-1, RANTES & KC). It may be that elevated levels of IL-6 may have been released directly from the muscle into the circulation; hence, the whole muscle content remained unchanged.

6.5.3 The effect of systemic administration of TNF-α on circulating serum cytokine levels.

The presence of elevated cytokines in circulation in response to TNF-α treatment is well defined. Furthermore, elevated levels cytokines in circulation have been correlated with the development and pathology in diabetes, cardiovascular disease and sepsis (Pedersen, 2009b). The precise source of cytokines in these states was previously thought to be associated with various sub-sets of lymphocytes. However, the discovery that skeletal muscle can act as a source of cytokines has provided a new perspective on the aetiology chronic inflammatory states.

TNF-α treatment of mice resulted in significant elevation in IL-6, MCP-1, RANTES, IL-1β and KC levels in serum compared with saline treated control mice (Figure 6.4). This was associated with elevations in muscle content of IL-6, MCP-1, RANTES and KC (Figure 6.3).

The detection of IL-1β in circulation following TNF-α treatment was of great interest. Our previous work *in vitro,* shown in Chapter 5, indicated there was no change in IL-1β gene expression or release of the IL-1β protein in response to TNF-α. The lack of expression of IL-1β protein in our *in vitro* model and in whole muscle *in vivo,* but, detection in circulation suggests that IL-1β is produced from an

alternative cellular source. Furthermore, a significant body of literature suggests the primary source of IL-1β *in vivo* is monocytes and macrophages (Rubartelli et al., 1990). Interestingly the presence of IL-1β in circulation is intriguing since it lacks a classical leader sequence for golgi-mediated release from cells it has been reported to be released by non-classical means – possibly via exosomes (Rubartelli et al., 1990).

The correlation in muscle and serum elevated cytokines provides the suggestion that muscle cytokine elevation may have a contribution to the levels of cytokines present in circulation. However, analysis of cytokine release from individual muscle fibres in response to TNF-α would provide a definitive conclusion.

6.5.4 The effect of treatment with TNF-α on cytokine release from isolated single muscle fibres from adult C57Bl6 mice.

The data presented in this chapter have demonstrated an elevation in muscle and circulating cytokine content in response to TNF-α treatment *in vivo*, and that skeletal muscle *in vivo* may be a significant contributor to the systemic inflammatory cascade.

To assess the contribution of muscle to serum cytokines directly we examined cytokine release from isolated fibres in response to TNF-α treatment. Significant increases in release of IL-6 and KC was observed from isolated single muscle fibres, in response to TNF-α treatment (Figure 6.5). No significant elevation was observed in MCP-1 and RANTES (Figure 6.5). IL-6 protein was readily detected in whole muscle tissue (Figure 6.3) and was elevated in circulation (Figure 6.4) following TNF-α treatment. Taken together with the release of IL-6 at the single fibre level

(Figure 6.5) in response to TNF-α, these data confirm muscle as a significant contributing source of IL-6.

It has previously been demonstrated that IL-6 induces skeletal muscle atrophy by down-regulation of ribosomal S6 kinase phosphorylation (Haddad et al., 2005). Thus, in a hyperinflammatory state muscle-derived IL-6 may exacerbate the inflammatory cascade and contribute to the organ dysfunction and perhaps mortality in these individuals. Previously it has been reported that adipose tissue accounts for approximately 30% of circulating IL-6 (Mohamed-Ali et al., 1997). Data here suggest muscle may have an equal or even greater role in contributing to circulating levels of IL-6.

Data in Figure 6.4 show that treatment of adult mice with TNF-α cause significant elevation in the circulating levels of IL-6, 3 hours following treatment (250pg/ml ± 53.23). Data shown in Figure 6.5 show significant increased release of IL-6 from isolated muscle fibres of adult mice, following 3 hours treatment with TNF-α (0.31pg/mg ± 0.02). Based on the average weight of an adult mouse at approximately 20g we can assume total muscle weight to be around 2g and total blood volume approximately 1ml. Therefore, we can calculate that skeletal muscle releases approximately 620pg of IL-6 over a 3 hour period. Studies in rodents have demonstrated following infusion of IL-6 that is cleared by approximately 80% per hour following treatment (Deaciuc et al., 1996). Thus, we hypothesise that over the 3 hour period we can expect to see a reduction in 620pg of IL-6 released from muscle to around 125pg. Compared with the 250pg we record in circulation at 3 hours data suggests that muscle contributes approximately 50% of total IL-6 in circulation, following treatment with TNF-α. Thus, this suggests that muscle is a significant contributing source of circulating IL-6.

Release of KC at the single-fibre level, coupled with elevations in the circulation suggests skeletal muscle is a significant source of KC. KC is better known as Gro-α in humans, and is a key neutrophil chemoattractant. Although the precise role of muscle-derived KC is poorly understood we hypothesise that it may play a role in neutrophil recruitment to muscle and the subsequent elevations in ROS which contribute to muscle dysfunction in the critically ill (Callahan et al., 2001).

Data in Figure 6.4 show elevated circulating levels of KC, following TNF-α treatment at 3 hours (9.61pg/ml ± 3.44). Data shown in Figure 6.5 show significant increased release of KC from isolated fibres, following TNF-α treatment (0.49pg/mg ± 0.28). Based on total muscle mass of an adult mice and clearance rates mentioned previously we can hypothesise that muscle release approximately 980pg of KC, over a 3 hour period. Taken into account the rate of clearance, this would suggest that muscle contributes approximately 588pg of KC into circulation. This value far exceeds the observed concentration of KC at 9.61pg/ml; this discrepancy may be due to several factors. Rates of clearance of circulating molecules, in particular, cytokines are a complex and poorly understood area. Thus, it is likely they differ greatly, suggesting KC may be metabolised from circulation at a much faster rate. However, our data do suggest muscle is a key source of KC in circulation.

The lack of response in MCP-1 and RANTES released from isolated single fibres suggests that although TNF-α up-regulated levels in the circulation, skeletal muscle is not the primary source of these cytokines *in vivo*. However, the detectable release of MCP-1 and RANTES from muscle *in vivo* supports our data in Chapter 5 and other studies, which demonstrated MCP-1 release from C2C12 myotubes (Henningsen et al., 2011). These data suggest MCP-1 and RANTES may harbour a more local role, signalling to neighbouring muscle fibres, bone, connective or

adipose tissue. No significant release of MCP-1 or RANTES was observed following treatment of isolated fibres with TNF-α. Thus, data suggest that elevated circulating levels present following treatment of mice with TNF-α are derived from a non-muscle source.

6.5.5 The effect of ageing on the ability of skeletal muscle to act as source of HSPs and cytokines.

As we age our susceptibility to infection or trauma is markedly increased, which is characterised by the cellular senescence throughout tissues and organs in the body. In terms of skeletal muscle we see a progressive loss of muscle mass and function as we age. The age-related loss of muscle mass and functions is a significant confounding factor in the critically ill, as is one of several predictors of mortality (Griffiths, 1996). In ageing our ability to respond to stress is blunted, as we have demonstrated in the lack of response by HSPs (McArdle et al., 2004). Moreover, we experience a persistent state of low-grade systemic inflammation (Pedersen et al., 2000, Krabbe et al., 2004). The impact of ageing on the locomotive aspect of skeletal muscle is well defined; however, given the immunogenic function of muscle we hypothesise ageing will have a deleterious effect on this function also.

We examined the effect of ageing on the immunogenic function of muscle, firstly by examining the ability of muscle to release cytokines. Data show significant release of IL-6 and KC from fibres from old mice, in response to TNF-α treatment (Figure 6.6). No change was observed in levels of MCP-1 and RANTES released (Figure 6.6). Data indicates that the ability of skeletal muscle to release cytokines in response to TNF-α is not affected by ageing. Data showed a comparable response to fibres taken from adult mice (Figure 6.6). A reason for this observation could be due to the

muscle fibres being taken out of the low-grade inflammatory milieu *in vivo* and placed into an inert cell culture environment. Thus, the inflammatory environment surrounding muscle could be the defining factor in how muscle behaves as an endocrine organ.

Given the aberrant inflammation present during ageing, we hypothesised that control untreated muscle fibres would exhibit a basal increased release of cytokines. Although detectable levels were present in the media from control fibres the levels detected were again comparable to the adult mice (Figure 6.6). These data suggest that aberrant low-grade inflammation in the elderly is not primarily due to cytokines release from skeletal muscle or is modified by optimising the external environment.

Typically the heat shock response is blunted during ageing (McArdle et al., 2004). Data has shown previously, in fibres from adult mice, that they release HSP60 a known extracellular signalling molecule in response to TNF-α (Figure 6.2). The release of HSPs has been described as means of early defence, acting as danger signals to the immune response (Zanin-Zhorov et al., 2005). Thus, we hypothesise that the dampened intracellular heat shock response during ageing is applicable to eHSPs also. Data demonstrated no significant release of HSP60, from fibres in response to TNF-α, indicative of the blunted HSP response as a result of ageing (Figure 6.7). These data suggest that the dampened eHSP release from muscle may contribute to the difficulty in dealing with infection and trauma experienced during ageing. However, the precise function of eHSPs on the whole *in vivo* remains poorly understood.

6.6 SUMMARY

Data shown in this chapter demonstrate that treatment of adult mice in TNF-α causes up-regulation in muscle content of HSP70 and specific release of HSP60 from isolated muscle fibres. Supporting data shown in Chapter 3 which demonstrated that muscle can release HSP60. However, based on the levels of HSPs detected in the circulation published in the field, data suggest muscle is not a significant source of eHSPs.

Treatment of adult mice with TNF-α caused elevation in muscle content and in tandem with elevations in serum content of IL-6, MCP-1, RANTES and KC. Examination of the effect of TNF-α on isolated fibres from adult mice demonstrated muscle fibres release a significant amount of IL-6 and KC. Moreover, data suggest that muscle is a significant source of circulating IL-6 and KC.

Studies using fibres taken from old mice show a suppressed release of HSP60 in response to TNF-α treatment compared with fibres of adult mice. However, there was no significant difference in patterns of cytokine release between adult and old mice.

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 SUMMARY OF MAJOR FINDINGS

1. Treatment of C2C12 myotubes with TNF-α resulted in up-regulation of HSPs in muscle cells and the specific release of HSP60 via exosomes.
2. Treatment of C2C12 myotubes with TNF-α resulted in up-regulation of the expression of several inflammatory cytokines, and the specific release of IL-6, MCP-1, RANTES and KC via golgi-mediated processes.
3. Treatment of C2C12 myotubes with HSP60 induced activation of NFκB in cells and the release of IL-6, MCP-1 and RANTES. The precise role of muscle-derived exosomes remains unclear.
4. Treatment of adult mice with TNF-α resulted in increased muscle content of HSP70. Treatment of isolated muscle fibres with TNF-α resulted in specific release of HSP60, although data suggest muscle is unlikely to be a significant source of HSPs in pathological conditions such as systemic inflammation.
5. Treatment of adult mice with TNF-α resulted in significant elevation in muscle content and serum levels of the cytokines IL-6, MCP-1, RANTES, and KC. Treatment of isolated muscle fibres with TNF-α resulted in significant release of IL-6 and KC, suggesting that muscle is a significant source of cytokines during systemic inflammation.
6. Studies on muscle fibres isolated from old mice demonstrated that, following treatment with TNF-α, a suppressed release of HSP60 occurs, compared with fibres of adult mice. In contrast, patterns of cytokines released from old fibres are comparable to that from fibres of adult mice.

7.2 GENERAL DISCUSSION

The hypothesis of this thesis was that:

Exposure of skeletal muscle to TNF-α induces a specific pro-inflammatory profile in muscle and the release of specific cytokines by muscle. Moreover, that muscle is the major contributing source of cytokines *in vivo*. Skeletal muscle can act as a novel source of eHSPs and exosomes which have specific signalling properties, which can signal to neighbouring tissues. Overall, muscle is a primary contributor to endocrine signalling molecules which may exacerbate the overall hyper-inflammatory state in the critically ill. Moreover, the ability of muscle to act as an endocrine organ is attenuated during ageing.

7.2.1 The ability of skeletal muscle to act as a source of eHSPs

Data in Chapter 3 show that muscle cells specifically release HSP60 in response to treatment with TNF-α. Moreover, release of HSP60 occurred via the release of exosomes. Examination of the proportion of HSP60 associated with exosomes determined that approximately 70% of HSP60 released from C2C12 myotubes was via exosomes (Chapter 5). Study of fibres isolated from adult mice reinforced these *in vitro* findings, whereby, TNF-α treatment of fibres resulted in a significant release of HSP60. Examination of the contribution of the HSP60 released to overall circulating levels reported in the literature suggests that muscle is not the primary source of HSP60 in circulation (Chapter 6). The findings that skeletal muscle can release HSP60 supports prior studies in cardiac muscle, which demonstrated exosomal release of HSP60 in response to stress (Kim et al., 2009). In a large cohort study the levels of HSP60 released by muscle *in vivo* account for only around 25-30% of serum levels (Shamaei-Tousi et al., 2006). The work carried out in this thesis

is the first to address the origin of eHSPs *in vivo*. The observation that muscle is not the predominant source of eHSPs is interesting, given that muscle is the largest protein store in the human body. However, only a small number of studies have measured eHSPs in circulation. Furthermore, our data suggest that the majority of muscle-derived eHSPs are released in a specific manner via exosomes. It is unclear whether the levels recorded in circulation by several groups are comprised solely of exosome-bound HSPs, which implies a specific signalling process or whether the eHSPs are not compartmented in serum, suggesting a non-specific release as a result of apoptosis/necrosis. The latter suggests that eHSPs in circulation may be an indicator of severe pathology; several studies support this, correlating sepsis (Wheeler et al., 2007) and cardiovascular disease with elevated eHSPs (Pockley et al., 2003). However, it is likely that exosome-bound HSPs do contribute, albeit a small proportion, to the overall circulating pool. The presence of HSPs within exosomes has been described by numerous studies on exosomes, derived from a large variety of cell/tissue types (Clayton et al., 2005) and bodily fluids (Lasser et al., 2011). Conversely, significant levels of eHSPs have been reported in the serum of healthy individuals (Pockley et al., 1999). This finding is paradoxical to the overall "danger-signal" paradigm associated with the presence of eHSPs in circulation (Breloer et al., 2001). There is a lot of variation between levels of eHSPs in circulation between individuals, with some research indicating there may be a genetic component which determines this, however this area still remains poorly understood (Shamaei-Tousi et al., 2007b). Thus, it may be difficult to associate circulating HSP levels with severity of illness in pathological conditions, the precise function of eHSPs *in vivo* remains unclear.

The study of HSP60 release from fibres isolated from old mice following treatment with TNF-α showed an attenuated release of HSP60, compared with fibres from adult mice. We hypothesised that old muscle would have an aberrant production and release of HSP60 in response to elevated TNF-α levels, which contributes to the inflammatory environment seen in ageing. Our data support findings that the HSP response is blunted during ageing (Vasilaki et al., 2002). Thus, these data suggest that the dampened eHSP response may be a contributing part of the immunoscenesence seen during ageing, and the impaired ability to respond to insult. Moreover, these data perhaps highlights that the therapeutic targeting of the HSP response e.g. glutamine supplementation to upregulate the cytoprotective response in muscle of old mice, may play a role n maintaining the immunogenic function of HSPs.

7.2.2 The immunomodulatory properties of muscle-derived eHSPs and exosomes

The precise function of extracellular HSPs is poorly understood. Significant research has suggested that eHSPs may act as danger-signals, released by cells and tissues under stress, to communicate to neighbouring or distal cells to provoke a response. However, the majority of research which has reported the immunostimulatory functions of eHSPs has been carried out *in vitro*, typically using supraphysiological concentrations of HSPs (Zhao et al., 2007). Our study used physiological concentrations of eHSP60 and these induced activation of NFκB in muscle and the production and release of cytokines from muscle. Overall, our data support the immuomodulatory function of eHSPs that has been widely reported (Asea, 2006). Thus, elevation in circulating levels of HSP60 in the critically ill may have profound

effect, inducing cytokine release and exacerbating the overall inflammatory state. However, it is likely that the HSP60 is derived from a non-muscle source, such as cells of the immune system. Therefore, targeting of circulating eHSP60 may be an avenue for therapy in the critically ill resulting in dampening of the hyper-inflammatory state.

Although muscle is not a primary source, levels occurring physiologically are still capable of up-regulating cytokine release *in vitro*. Thus, the question arises, what prevents the levels of HSP60 in circulation from inducing a similar response *in vivo*? The occurrence of significant levels of circulating eHSP60 in healthy individuals suggests that eHSP-induced cytokine release must be regulated. HSP60, like all members of the HSP family, is promiscuous by nature, with a unique ability to bind a wide array of proteins. However, HSP60 has a unique affinity for its chaperonin complex partner, HSP10. Like HSP60, originally thought to be primarily mitochondrial associated, HSP10 is also located in circulation (Cavanagh and Morton, 1994). Unlike eHSP60, eHSP10 has been characterised to be anti-inflammatory (Johnson et al., 2005). Thus, we hypothesise that HSP10 readily binds HSP60 in circulation, creating the chaperonin complex, which is then unable to bind receptors and thus elicit up-regulation of cytokines in healthy individuals. Therefore, we hypothesise that it is only free existing HSP60 in circulation that has an immunomodulatory effect. Thus, a measure of both circulating HSP10 and HSP60 may be a more accurate predictor of inflammatory pathologies. However, no studies have investigated circulating HSP10 in healthy and individuals with pathology. Our hypothesis on the anti-inflammatory effect of HSP10 is supported by studies in our laboratory that demonstrate that overexpression of HSP10 in mice suppressed release

of TNF-α from muscle fibres exposed to LPS (Kayani et al. 2011 – in preparation). It may be that HSP60 levels in circulation is a naturally occurring biological process, however in the critically ill the non-specific release of HSP60 due to cell death may favour a more pro-inflammatory environment. Levels detected in the circulation of healthy individuals are capable of inducing a significant inflammatory response *in vitro* (Chapter 4). Thus, we hypothesise that a mechanism must be in place to regulate the chaperokine function of eHSP60 *in vivo* to maintain homeostasis. This process could potentially involve eHSP10, where by the chaperonin complex is formed in the extracellular environment, which then suppresses the stimulatory ability of eHSP60 due to a conformational change in its tertiary structure.

Our study of the immunomodulatory capacity of exosomes, based on their HSP60 content showed no effect on muscle or on alternate tissue such as bone. The levels of HSP60 detected in exosomes is a fraction of the content reported in serum in a variety of models and our data suggest that the levels detected in exosomes are insufficient to invoke cytokine release directly (Figure 5.2). Thus, we hypothesise that exosomes play an alternate role. Several studies on the biological function of exosomes describe their primary role as means of genetic transfer of both mRNA and microRNA (Valadi et al., 2007). Although muscle-derived exosomes contain HSP60, we believe this is not of any biological significance. Exosomes may be involved in genetic transfer, between neighbouring muscle fibres, the neuromuscular junction or more distal cells and tissue. However, currently this area is poorly understood and beyond the scope of this Ph.D.

7.2.3 The ability of skeletal muscle to act as a significant source of cytokines

The focus of most of the work carried out to date on muscle-derived cytokines is exercise. This thesis is one of the first studies to examine the effect of inflammation on the pattern of cytokine release from muscle. Our study is also the first to examine cytokine release from isolated single muscle fibres. The majority of work published on myokines reports primarily, changes in gene expression using muscle biopsies, in association with immunohistochemistry images and aterio-venal and plasma measurements. However, there are some limitations to these techniques, the use of immunohistochemistry to accurately localise cytokines in muscle fibre cross-sections is difficult – most images show a typical saturation throughout the image. Furthermore, the use of aterio-venous measurements does not precisely identify the tissue of origin. Thus, cytokines may be released from a whole host of cellular origins, such as endothelial cells, adipose tissue, bone, satellite cells and tissue residing macrophages. In addition, circulating levels may be derived from a variety of immune cells or tissues in response to exercise. Thus, this underlies the importance of our data, which categorically demonstrates cytokine release from muscle cells and mature muscle fibres.

Exposure of C2C12 myotubes to TNF-α caused up-regulation in an array of pro-inflammatory genes, consistent with previously published data (Bhatnagar et al., 2010). The pattern of cytokine release seen *in vitro* was replicated *in vivo*, via the release of IL-6, MCP-1, RANTES and KC. More importantly, data indicate that muscle is a significant circulating source of IL-6 and KC. This is the first study to describe KC as a key myokine. Our data reinforce research published reporting muscle as a major source of IL-6. Our data indicate that the pro-inflammatory

environment in the critically ill can invoke cytokine release from muscle, which in turn may exacerbate the hyper-inflammatory state, contributing to muscle dysfunction.

The up-regulation of IL-6 and various anti-inflammatory components following exercise may provide an avenue for therapy in the critically ill. Physical exercise therapy of patients to promote IL-6 and the anti-inflammatory response may help to adjust the balance of a pro-inflammatory profile of cytokines released from muscle driven by TNF-α. Studies have demonstrated that patients in ICU which undergo a regime of physical augmentation have a better clinical outcome (Schweickert et al., 2009).

Examination of fibres isolated from old mice showed a similar pattern of cytokine release to that of adult fibres, when exposed to TNF-α. Our data indicate that ageing has no impact on the ability of muscle to release cytokines. This is an interesting observation, since immunoscenesence is a characteristic component of ageing. However, since the fibres are excised from old mice and placed into an inert environment our data support the theory that the systemic environment is a key determinant of muscle dysfunction in ageing (Conboy and Rando, 2005). Thus, these data support the role exercise-induced anti-inflammatory response to combat the aberrant inflammatory environment accrued during ageing

7.3 FUTURE DIRECTIONS

7.3.1 Mechanisms that control muscle-derived cytokine release: implications for the critically ill.

Control and therapy of the therapy of the hyper-inflammatory state in the critically ill has been the primary goal in an effort to combat sepsis over the last 20 years. Numerous clinical trials, primarily focused around anti-TNF-α therapy have largely proved to be ineffective, and in some cases exacerbate the problem. TNF-α appears to be a key regulator of the pro-inflammatory immune response, and has roles in numerous signalling pathways. Thus, targeting components downstream to modulate cytokine release from muscle may be a means of suppressing or reducing the hyper-inflammatory state in the critically ill. Furthermore, targeting of suitable patients is difficult and often the hyper-inflammatory state is well established before interventions have been administered. Myokine release occurs in tandem with activation of NFκB, which is a ROS sensitive transcription factor. Thus, we hypothesise that, in a similar manner to that proposed by (Li et al., 1999) myokine release is likely to be ROS mediated. Examination of cytokine release from muscle in the presence of specific blockers of ROS production e.g. SS31 – mitochondrial ROS generation would provide a greater insight into the mechanism involved and any avenues for therapy.

7.3.2 The anti-inflammatory function of eHSP10, role in the critically ill.

The use of eHSP10 in clinical trials for rheumatoid arthritis (RA) (Vanags et al., 2006) has demonstrated its ability to suppress the inflammatory response. Based on the success of clinical trials in RA, through the dampening of the inflammatory response, HSP10 may improve muscle function and survival in the critically ill.

Thus, we hypothesise that using a rodent model of sepsis, administration of HSP10 will help suppress the hyper-inflammatory state and improve outcome at both the muscle and whole-body level.

7.3.3 The functional role of muscle-derived exosomes in cellular communication and genetic transfer

The precise function of muscle derived exosomes remains poorly understood. Exosomes are an abundant source of mRNA and miRNA. Thus, profiling of the genetic components of muscle-derived exosome will provide an insight into possible functions. Examination of the genetic transfer from muscle fibres neighbouring muscle fibres and satellite cells would provide a better understanding of the intricate intercellular signalling processes which may play a role in myogenesis and muscle regeneration and how these processed are affected during ageing.

CHAPTER EIGHT

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