Investigating the impact of extracellular heat shock proteins on skeletal muscles; a potential role in muscle dysfunction in the critically ill?

Ali Hasan Chaudhry

2022

Investigating the impact of extracellular heat shock proteins on skeletal muscles; a potential role in muscle dysfunction in the critically ill?

Ali Hasan Chaudhry

A thesis submitted in fulfilment of the requirements of Manchester Metropolitan University for the degree of Master of Science (By Research)

Department of Healthcare Science

Manchester Metropolitan University

2022

Declaration:

All data presented in this report is my own work, furthermore no parts of this report have been copied. I understand that any plagiarism the use of unacknowledged third-party data will be dealt with seriously.

Signed: Ali Hasan Chaudhry

Acknowledgements:

I would like to express my sincerest gratitude to Dr Adam Lightfoot and Dr Nina Dempsey for supporting me throughout the period of this project. The covid-19 pandemic did impose delays upon the project however with the support and guidance of Dr Adam and Dr Nina, I was able to complete this project, despite falling severely ill for a lengthy period of time during this time.

Table of Contents

Abstract

An ongoing battle in long term hospital care is the onset of ICU acquired weakness, several studies have reported the elevated risk of ICUAW in cohesion with pre-existing medical conditions. Skeletal muscle dysfunction is a hallmark of ICUAW, with several studies reporting mitochondria can play a significant role; however, the mechanisms responsible are poorly characterised. Damage associated molecular patterns (DAMPs) are a broad classification of cellular components, which can modulate inflammatory pathways and are highly prevalent in chronic inflammatory conditions (e.g., sepsis) - one example of this is heat shock protein (HSP) 60. HSP60 functions as a molecular chaperone when in the intracellular and a cytokine when in the extracellular environment. We reasoned that eHSP60 may be relevant to muscle dysfunction in sepsis and looked to evaluate this capability in a pre-clinical in vitro model.

This study aimed to evaluate the impact of Extracellular Heat Shock Proteins on C2C12 cells, illustrating the potential effect of, eHSP70 and eHSP60 specifically, on the mitochondria and the effectiveness of TAK242 as an inhibitor of TLR4 which enables the production of lipopolysaccharide induced inflammatory mediators. C2C12 cells were treated with increasing concentration of eHSP70 and eHSP60 (5ng/ml, 10ng/ml, 25ng/ml, and 50ng/ml), with or without TAK242, followed by a 24-hour incubation period. U937 conditioned media was acquired in order to mimic an environment similar to that of an ICU patient, this was done by treating U937 cells with 100ng/ml of LPS for 1 hour followed by 1, 2, 4, 24-hour recovery period, the supernatant was collected and used to treat C2C12 cells.

Cells were counted using a Haematocytometer and quantified using WST-8 assay, assessing for cell viability. Mitochondrial function was assessed using Seahorse Extracellular Flux Analysis. Cell viability was seen to reduce when treated with increasing doses of eHSP70 and eHSP60, a significant reduction seen when treated with 5ng/ml of eHSP60.

Overall parameters of seahorse extracellular flux analysis were seen to increase overall.

1.0 Introduction

1.1 Skeletal muscle and skeletal muscle weakness

Making up 40% of total body mass and 50% of total body weight, skeletal muscle is a vital organ that is crucial for many processes including maintaining body posture, moving objects, initiating the swallowing reflex and enabling the breathing mechanism (Rome, Forterre, Mizgier and Bouzkari, 2019). Skeletal muscles are striated in appearance due to the arrangement of actin and myosin filaments, which combine to make a myofibril, which form skeletal tissue (Finn *et al,* 1996). Due to skeletal muscle mass being present throughout the body and over-seeing many important bodily functions, diseases affecting skeletal muscle mass can quickly become life-threatening especially when taking into consideration their ability to alter thoracic volume which allows the lungs to fill with air (Dave, Shook and Varacallo, 2021). There are several diseases which may affect skeletal muscles, and this can be seen by the manifestation of Skeletal Muscle Atrophy. Skeletal muscle atrophy is seen in long-term ICU patients but can also be caused by chronic disease such as heart failure, lung disease, sepsis and dystrophies (Fanzani et al., 2012). Skeletal muscle atrophy is characterised by deregulation of protein synthesis and pathways inciting degradation of cells (Krasnoff and Painter, 1999). The lack of muscle usage in critically ill patients, along with the reduced hydrostatic forces and pressures on the skeletal muscles results in skeletal muscle atrophy. In the setting of ICU, skeletal muscle atrophy is referred to as ICU-acquired muscle weakness (ICUAW) and presents as symmetrical limb weakness, seen largely in proximal muscles (Hermans and Van den Berghe, 2015). The exact incidence of ICUAW is unknown due to a wide variation in the patient population, risk factors, time of assessment and diagnostic used for criteria; however, it is believed the ICUAW can affect more than half of ICU patients. ICUAW is directly correlated with increased rates of mortality and can have profound impact on the health-related quality of life post-discharge. The combined effect of this can be seen on the healthcare systems as there an increased demand for resources during the stay and after being discharged from the ICU (Herridge, 2002).

Despite being a common illness seen amongst ICU patients, there are very few treatments and certainly non that are effective in preventing the onset of ICUAW in those patients whom it is known will be bed-ridden for long periods of time. Treatments such as insulin therapy and electrical muscle stimulation have been used however, the use of insulin therapy runs the risk of hypoglycaemia (Intensive versus Conventional Glucose Control in Critically Ill Patients, 2009), however a study conducted by Dirk *et al.,* found that electrical stimulation was seen to be an effective intervention strategy preventing skeletal muscle atrophy (Dirks et al., 2014). On conclusion, the study found no

muscle atrophy in the treated leg on the final day, however a steady decline of type of 1 and type 2 was apparent upon investigating biopsies acquired on the first and final day of the trial. Electric muscle stimulation is deemed to be cost-effective, and convenient as nursing staff can easily apply it to sedated patients. No adverse effects have been registered to date (Meesen et al., 2010)

ICUAW is divided into three components: critical illness polyneuropathy (CIP), critical illness myopathy (CIM) and critical illness neuromyopathy (CINM), however CIP and CIM can be present simultaneously and cannot be distinguished clinically when together (Stevens et al., 2009). CIP manifests in the form of symmetric, distal sensory-motor axonal polyneuropathy affecting limb and respiratory muscles, while also impacting sensory and autonomic nerves (Bolton, 2005). The common understanding is that CIP is caused by peripheral nervous system organ failure resulting through common systemic inflammation-induced pathophysiological processes such as reduced oxygen and nutrient delivery due to macro and microcirculatory impairment caused by myocardial depression, endothelial dysfunction, and vasodilation (de Letter et al., 2001). Another mechanism causing CIP is disruption of mitochondrial oxygen utilisation and ATP generation caused by reactive oxygen species and inhibition of mitochondrial respiratory chain (Latronico et al., 1996). CIM is presented by limb and respiratory muscle weakness, however, does not affect sensory and autonomic nerves. CIM symptoms result from alterations of functionality such as membrane inexcitabilty caused by a combined effect of depolarisation of resting membrane potential and hyperpolarisation of inactivation of sodium channels. Other contributing factors are decreased contractile protein function, mitochondrial dysfunction caused by reduced mitochondrial enzymatic activity and disruption of the respiratory chain function resulting in a significant reduction in oxygen utilisation and ATP production (Appleton and Kinsella, 2012).

1.2 Proposed molecular mechanisms of ICU-acquired muscle weakness

Protein synthesis and breakdown is crucial to allow rapid responses to differing demands, however the balance of protein synthesis and breakdown is altered in ICUAW. In a normal, healthy state, the balance of protein synthesis and breakdown allows muscles fibres to be repaired while also releasing amino acids to maintain gluconeogenesis (Kohlmeier, 2015) Muscle protein synthesis is seen to increase when muscles are subject to intensive exercise. Furthermore, the availability of amino acids impacts MPS as the amino acid leucine works best to stimulate MPS (Biolo et al., 1997. In ICUAW, it has been demonstrated that this balance is shifted to a catabolic state, and therefore the rate of muscle fibre degradation is greater than protein synthesis. A highly complex and specific process known as the Ubiquitin proteasome pathway (UPP) is the main mechanism of protein degradation.

The process is characterised by ubiquitin moieties marking the protein to be degraded, allowing the proteosome to target that protein and break it down into individual amino acids. Three enzymes play specific roles in breaking down the protein; E1 is the ubiquitin activating protein, E2 is the ubiquitin carrier protein and E3 breaks down the ligation of ubiquitin to specific proteins. Collectively, E1 and E2 are responsible for preparing ubiquitin for conjugation (Lecker, 2003). As myosin and actin filaments are highly packed together, UPP cannot hydrolyse entirely. However, Calpain and Caspase-3 are activated during muscle disuse and seen in high concentrations in the diaphragm and limb muscles. The two enzymes are able to bind and decompose proteins from the cytoskeleton causing actin and myosin to be released into the extracellular matrix; allowing UPP to complete hydrolysis by muscle specific E3 ubiquitin (Levine et al., 2011). UPP is upregulated due to several factors such as inflammatory proteins, increased superoxide and reactive oxygen species (ROS), stress stimuli and immobility. Interleukin-1β , a pro-inflammatory cytokine upregulated during inflammation and trauma, has demonstrated the ability to incite the expression of the ubiquitin gene, causing increased skeletal muscle catabolism (Wolfe, 2005).

Ubiquitin ligases can be expressed specifically in certain tissue. In the case of muscle tissue atrogin and muscle ring finger-1 (MuRF-1) are seen to be expressed specifically is muscle tissue. MuRF-1 in particular targets thick myosin filaments, and therefore could be the major factor in ICUAW (Ochala et al., 2011). Investigations have found that the UPP is induced in numerous atrophic conditions, amongst these are inactivity and inflammation, two common factors seen in ICU patients. The increased ubiquitination and degradation of IkB causes the release of the bound Nf-kB, a transcriptional factor responsible for regulating the expression of UPP proteins. IkB is an inhibitor of Nf-kB, interaction with IkB prevents the nuclear localisation sequence in the Nf-kB, consequently preventing nuclear translocation and keeping Nf-kB in an inactive state in the cytoplasm (Hayden and Ghosh, 2008). IkB is composed two catalytic subunits which are IKK-alpha and IKK-beta, and a regulatory subunit IKK-gamma. Another mechanism by which protein lysis occurs is autophagy-lysosome system, a basic mechanism regulating and disposing of damaged dysfunctional proteins. It therefore has a crucial role in maintaining cell homeostasis and degradation. Autophagy-lysosome, alongside the UPP, is regulated by Forkhead box O protein (FoxO) and mTOR (Mizushima et al., 2008). The dysregulation of what is a crucial mechanism in cell homeostasis, autophagy-lysosome leads to a large amount of protein degradation of muscle proteins, resulting in ICUAW. Mitochondrial damage can also stimulate proteolysis. The mitochondria are involved in several processes such as signalling, cell differentiation and most importantly energy production. Disuse and aging results in DNA

mutations in the mitochondria causing increased production of ROS, consequently damaging cellular components and hindering cellular and tissue functionality.

In contrast to up-regulated protein breakdown, significant reduction in protein synthesis is also seen to be a contributing factor to ICUAW, this being seen more commonly post-surgery where the patient has been advised bed rest. Reduced protein synthesis has been reported to remain for 3 days in many studies, although some studies have reported reduced protein synthesis up to 30 days (Petersson et al., 1990). Regardless of the time frame, the majority of the patients investigated were described to be in a catabolic state, whereby the amount of protein lysis was far greater than protein synthesis. Insulin-like growth factor 1 ((IGF-1)/ Akt signalling cascade is deemed to be responsible for the regulation of protein synthesis. IGF-1 binds to serine/theronine kinase receptor, activating the MAPK signalling cascade, increasing the myoblast proliferation; the resulting effect is the activation of mammalian target of rapamycin (mTOR) and inhibition of glycogen synthase kinase-3beta (Glass, 2003). Akt signalling is also responsible for phosphorylating FoxO, which allows the inhibition of MuRF-1 and atropin, both proteins known for their role in the UP-pathway (Stitt et al., 2004). Investigators found that IGF-1 is expressed upon contraction and reduced upon disuse, this could suggest the potential mechanism as to which protein synthesis is reduced.

The mechanisms which mediate muscle mass loss, whether that be increased proteolysis or decreased protein synthesis, are commonly caused by inflammation. One such phenomenon believed to incite Inflammation in ICU patients is a cytokine storm, a life threatening systemic inflammatory syndrome which involves high levels of circulating cytokines and increased activation of immune cells. A cytokine storm can be caused by several therapies, pathogens and cancers (Fajgenbaum and June, 2020). Cytokine storm refers to several disorders which present themselves through constitutional symptoms, systemic inflammation and multi organ dysfunctions which may rapidly lead to multiorgan failure if not treated (Lee et al., 2019). Amongst the mass of cytokines, three cytokines have been investigated in relation to critically ill patients; TNF-alpha, IL-1β and IL-6 (Friedrich et al., 2015). Investigators have found that IL-1 is able to bind to ryanodine receptor 1, which inhibits the release of calcium ions from the sarcoplasm; therefore, deemed to be a contributor to muscle weakness as Ca ions are required for muscle tissue contractions (Kraner et al., 2012). TNF-alpha cultured with myotubes presented with reduced myotube diameter and total muscle protein content (Li et al., 1998). Winkleman conducted a study in which involved the collection of blood samples from two groups: ICU patients and healthy controls. It was found that TNF-alpha levels were consistently high in ICU patients, in comparison to the control group. In line with this, the elevated levels of TNF-alpha

prove detrimental as they can incite mitogen-activated protein kinases, which increases the signalling to MuRF-1 and Atrogen-1, causing UPP-mediated protein degradation (Li et al., 2005). IL-6 was found to be elevated in myotubes treated with plasma from patients in septic shock, coinciding with findings of decreased myosin content and upregulating protein kinases MuRF-1 and atrogen (van Hees et al., 2011).

When cells undergo stress caused by ROS, specific mechanisms are initiated to limit the extent of the damage and allow the cell to recover. This protective mechanism is, in part, carried out by a group of high conserved proteins, termed Heat Shock Proteins (HSPs). Under normal conditions, HSPs function as molecular chaperones involved in general housekeeping. However, upon exposure to a stress stimulus, HSPs are upregulated to increase their chaperoning activity and thereby prevent protein aggregation which can ultimately lead to cell death (Rothman and Schekman, 2011).

1.3 HSP (intracellular) basic biology

HSPs were first discovered by Ritossa in 1962 (Ritossa, 1962), who reported a Drosophilia salivary gland chromosome puff which came about after exposure to high temperatures. Following this, numerous investigators have found that the heat shock response is highly conserved and ubiquitous, in all organisms, as it functions as a defensive mechanism against heat shock, inhibition of energy metabolism, oxidative stress and a wide range of other cellular insults (Sorger, 1991). Cellular stress causes the misfolding of important proteins, which are deleterious by nature (Jolly, 2000). Cells deal with this stress at a molecular level by inducing the synthesis of HSPs. These proteins can be categorised into two groups; (1) molecular chaperones which are responsible for the folding, translocation and refolding of intermediates; (2) proteases. One example of the role of proteases is seen in the ubiquitin-dependent proteasome which ensure damaged and short-lived proteins are broken down efficiently (Kriegenburg et al., 2012).

The two groups are then divided into families according to their molecular weight I.e, HSP100, HSP90, HSP70, HSP60, HSP40 and sHSP. Despite a long history of referring to these HSP families by this nomenclature, the sequencing of the human genome led to the identification of additional proteins within well-established HSP families. As such, a new nomenclature system was introduced in 2009 by Kampinga et al. The new nomenclature can be seen in Table 1.1. It should be noted though, that this nomenclature has not been widely adopted and many of the studies relevant to this thesis refer to the old naming systems. As such, this thesis will refer to Hsp60 and Hsp70, rather than HSPD1 and HSPA1A.

Table 1.1: Old and new nomenclature for HSPs.

Gene Name	Current Nomenclature	Original Nomenclature
HSPB1	HSPB1	Hsp27
DNAJB1	DNAJB1	Hsp40
HSPD1	HSPD1	Hsp60
HSPA8	HSPA8	Hsc70/Hsp73
HSPA9	HSPA9	Mortalin/ mtHsp70
HSPA1A	HSPA1A	Hsp72
HSPA5	HSPA5	GRP78
HSPC1	HSPC1	Hsp90
HSPH2	HSPH2	Hsp110

Members from each HSP family are either constitutively expressed or induced after a stress insult which may be directed towards different compartments of the cell. Such insults include hypo- and hyperthermia, UV radiation, pathogens and other forms of stress (Relja and Land, 2019). The HSP70 family is a good example of demonstrating complex growth-regulation and stress-induced gene expression patterns; Hsc70 and Hsp70 are cytosolic and nuclear, whereas Glucose related protein 78 (GRP78) is localised to the endoplasmic reticulum. mHsp70 is restricted to the mitochondria (Wu, 1995). Investigations on a molecular level have highlighted the significance of the Heat Shock Element (HSEs), a protein with multiple binding sites, essential for inducing the production of HSPs. HSEs are found upstream of transcription sites and require the binding of Heat Shock Factors (HSF) momentarily. Three HSF can be found inside human cells, all essential for inducing HSPs (Figure 1.1). HSF1 can be found in the cell constantly and has a primary role in regulating protein expression during proteotoxic stress conditions, despite being constitutively produced it remains inactive until a stress stimulus is present (Åkerfelt et al., 2010). HSF2 is specifically expressed during hemming-induced cell differentiation and inhibition of ubiquitin-dependent proteosome and finally, HSF4 is expressed in tissue specific manner and shows DNA-binding activity (Morimoto, 1998). HSPs play a key role in regulating the oxidative—antioxidant balance, with the aid of the antioxidant system to prevent and diminish the effects of ROS on cells. ROS are seen to be produced in several systems localised on the plasma membrane in the cytosol, peroxisomes and on the membrane of mitochondria and endoplasmic reticulum: all within the skeletal muscle cell (McCord, 1988). Several studies have found that Hsp70 up regulates the production of the 20S proteasome, allowing it to degrade oxidised

proteins, therefore preventing the accumulation of oxidised proteins which ensures the cell does not undergo necrosis. The significance of HSPs regulating the oxidant-antioxidant balance can be seen in a study conducted by Klumpen et al. which showed an increase in HSPs when ROS levels were elevated, enabling the HSPs to limit the damage inflicted on cellular components (Klumpen et al., 2016). HSPs may also bind to oxidised proteins in which case the surface expression of the HSPs is unregulated and can result in an autoimmune response (Profumo et al., 2018).

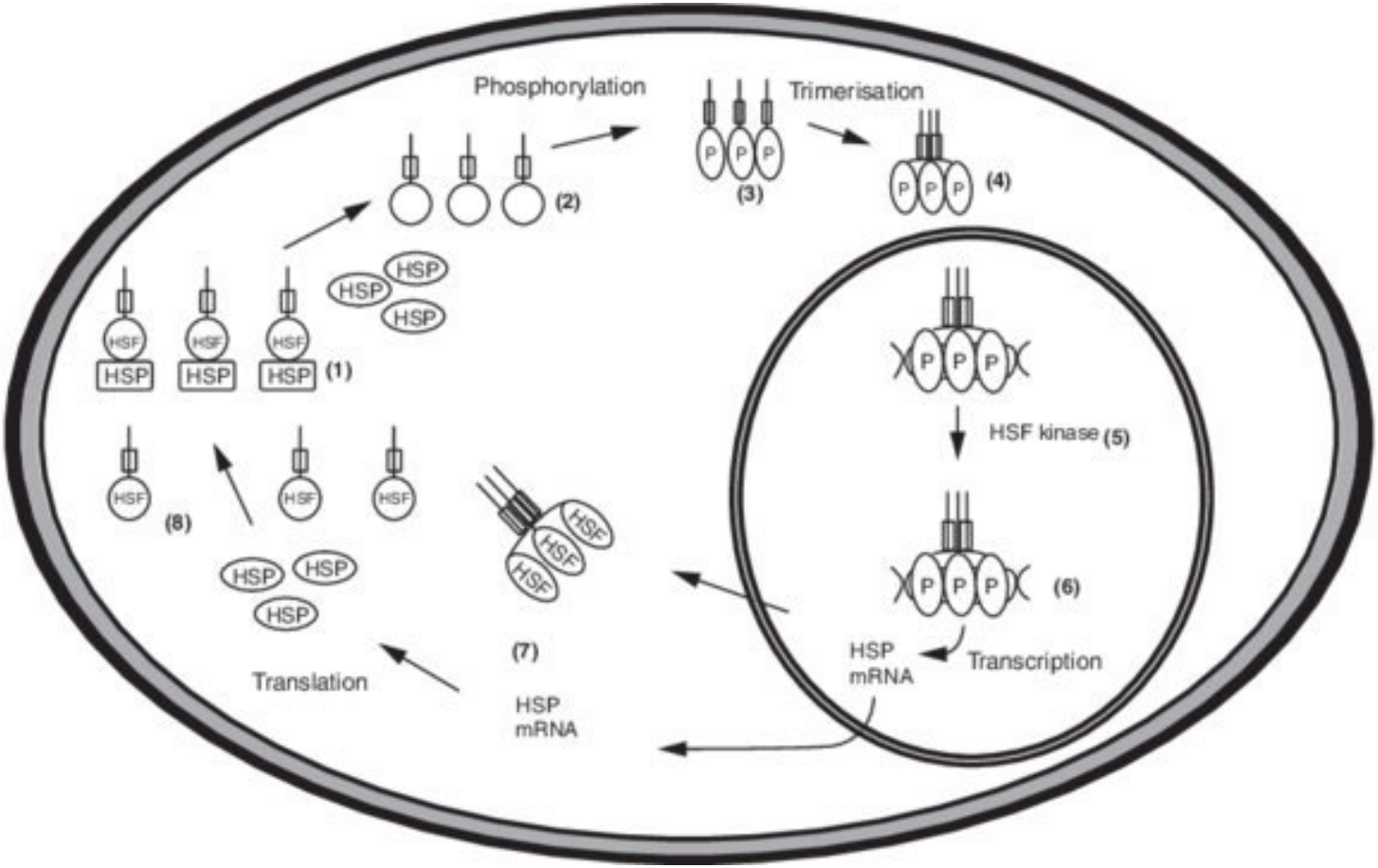


Figure 1.1: Stimulation of HSPs via HSP transcription and translation (Taken from, Tytell and Hooper, 2001). Heat shock factor (HSF) remains inactive by being bound to Heat shock chaperone (Hsc) or HSPs found in the cytoplasm, when a stress stimuli is received competition between unfolded protein and HSF leads to the unbinding of HSF from HSP. At this point it is phosphorylated by protein kinase C which promotes it to trimerise. The trimers then enter the nucleus, where further phosphorylation results upregulating binding to heat shock DNA, consequently stimulating the transcription of mRNA. The timers will then return to the cytoplasm and associate with HSP, granted enough additional HSP is produced.

The skeletal muscle is one such tissue that is continuously producing oxidative species, which are kept balanced with antioxidants. Antioxidants are endogenous or exogenous molecules that diminish all forms of oxidative stress or its consequences; this is achieved by an enzymatic and non-enzymatic system work synergistically preventing free radical damage. Antioxidative deficiencies can occur due to the lack of dietary intake, synthesis of endogenous enzyme or increased antioxidant utilisation (Kurutas, 2015). Oxidative stress can induce mitochondrial dysfunction, increase ubiquitin proteasome system activity, increase myonuclear apoptosis and decrease activity of the protein synthesis pathway causing skeletal muscle dysfunction (Gomes-Marcondes and Tisdale, 2002). A study found that intracellular HSPs have a cytoprotective role on skeletal muscles cells, after observing a short-term increase in levels of HSPs. This is indicative of a protective protocol in place to protect the skeletal muscle cells from contractile induced oxidative stress. The study found that contractile activity induced Hsp70 in the hindlimb muscle of young mice, however, a greater rise was found in Hsp60 which localised to the mitochondria. This would be indicative of the higher mitochondrial content of the *soleus* in comparison to the *extensor digitorum longus* (EDL) muscles (McArdle et al., 2001). Stimulating the levels of intracellular HSPs may enable an effective treatment for patients with sepsis-induced ICUAW. Singleton et al., (2005) found that glutamine led to an increased expression of Hsp70, which was seen to attenuate metabolic dysfunction. Wischmeyer et al. (2001) also found that glutamine induced Hsp70 and Hsp27, resulting in no animal deaths from induced sepsis after 20 hours. Therefore, there is compelling evidence for the of targeting HSPs in treatment for ICU acquired muscle weakness. However, the above refers to a seemingly protective role of intracellular HSPs within the muscle cells. Sepsis and other critical illnesses, however, also cause release of large concentrations of proinflammatory mediators and 'danger signals' including HSPs into the extracellular space, and the role of these extracellular HSPs in terms of muscle cyto-protection is less clear.

1.4 Extracellular HSPs (eHSPs) - basic biology and externalisation

The prevailing understanding of HSPs, for many years, was that they remain localised within the cell and are only released upon cell necrosis by a passive mechanism (Calderwood, 2007). This was due to the fact that HSPs lack a leader sequence which allowed for classical Golgi-mediated release. Therefore, the common understanding was that HSPs were responsible for intracellular functions. However, in the mid 1980's a team of researchers discovered that HSPs can in fact be released into the extracellular space. The group were studying newly synthesised glial proteins transferring into the axon, at which point they discovered one of the proteins was similar in molecular weight to Hsp70. Further investigation by the group reported an increase in Hsp70 and Hsp95 in the glial sheath and reduction of other proteins, furthermore the higher rate of transfer of Hsp70 and Hsp95 into the axon suggested the release to be a protective mechanism against metabolic stress and injury, suggesting

the cytoprotective role of HSPs in the extracellular space. Despite the lack of knowledge with regard to the mechanism of release, there seem to be several mechanisms. Hightower and Guidon, based on the observation that actin and another form of cytoplasmic protein is accompanying the HSPs, suggested that the mechanism of release is that HSPs could be released as a component in a group of proteins located in the sub-plasmalemmal network, suggesting the theory that membrane bound vesicles are shed from the outer membrane as seen in dendritic and tumour cells (Hightower and Guidon, 1989). In support of this, it was found that Hsp70 and Hsp90 are associated with the membrane micro domain which is found to be present during lipid rafts in the membrane (Chen et al., 2005), a direct correlation was found between the sorting of proteins into exosomes and lipid rafts (Petrov et al., 2017). Ion-conducing channels are another route for HSP release into extracellular space. This was demonstrated by Negulyaev et al (Negulyaev et al., 1996) who found that 30 - 100 ug of 2:3 mixture of Hsc70 and Hsp70 from bovine skeletal muscle, can activate K+ (potassium) channels in human promonocyte cells. In more recent times, it was found that HSPs can be released into the extracellular space in free form utilising the lysosomal pH gradient (Mambula and Calderwood, 2006). The majority of the findings suggest the most common mechanism of release into the extracellular space is through vesicles. Exosomes containing HSPs possess a wide range of properties, of which immmuno-stimulatory and immuno-suppressive are also included; this is determined by factors such as protein content of the exosome, origin and target cell (Gastpar et al., 2005).

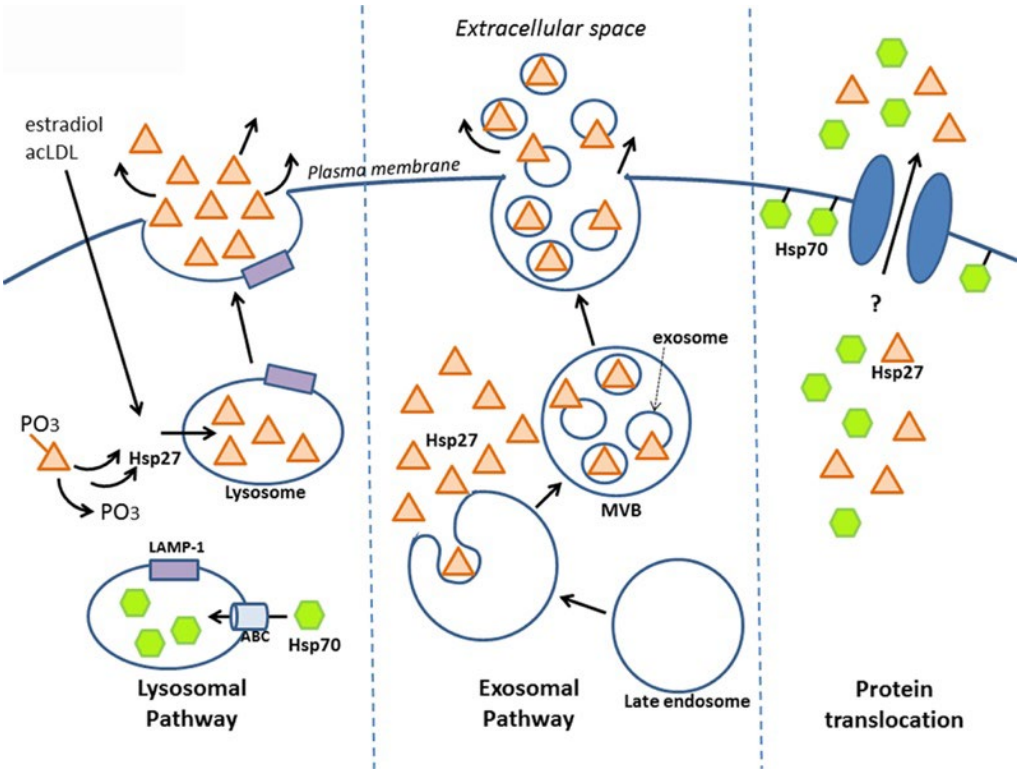


Figure 1.2: Diagram showing the proposed mechanisms of HSP release into extracellular environment (Taken from, Batulan et al., 2016). The diagram shows three, amongst several other, pathways HSPs may use to be released into extracellular space. In the lysosomal pathway, HSPs move into the lysosome through channels, the lysosome then fuses with the membrane and releases the HSPSs into the extracellular space. In the exosomal pathway, late endosomes engulf HSPs into exosomes and then travels to the membrane where they fuse with it, consequently releasing the HSPs into the extracellular space. The third mechanism is through protein translocation either through transporters such as ABC transporters, or by the direct association of HSPs to the cell membrane creating channels for HSPs to travel through.

When released into the extracellular space, the roles of HSPs depend on various factors as discussed before and by the receptors they bind to. The binding to receptors can either stimulate an immuno-stimulatory pathway or can cause an immuno-suppressive response. Cells undergoing stress or dying will release signalling molecules such as high ability group box 1 protein, initiating an innate immune response. The molecules released by the cells are referred to as damage associated molecular patterns (DAMPs) and several studies found eHsp70 to be DAMP, binding to toll-like receptor 2 and 4 (TLR2 and TLR4). This was displayed in the study conducted by Asea et al. in which eHsp70 bound to TLR-4 and increased the expression of tumour necrosis factor alpha (TNFα), interleukin 1beta (IL-1β) and IL-6 in human monocytes. The study also showed that eHsp70 can induce two different signalling pathways; one dependent on C14 and intracellular calcium, upregulating the production of IL-1β, IL-6 and TNF-α, and the other independent of CD14 but dependant on intracellular calcium, which increased TNF-α but no expression of IL-1β and IL-6 (Asea et al., 2000).

HSPs can also function as pattern-associated molecular patterns (PAMPs). PAMPs bind to the pattern recognition receptors on macrophages and dendritic cells, hence activating the innate immune system. Commonly, it is seen that the NKK and MAP-kinase pathways are activated when PAMPs bind to a PRR, most like toll-like receptor (TLR). This binding will activate inflammatory factors Nf-kB and stimulate the production of cytokines (Colaco et al., 2013).

eHSPs also play a role as pro-inflammatory factors, activating pathways to induce inflammation and killing of cells. A study conducted by Daniels et al (Daniels et al., 2004). found that elevated Hsp70 levels in targeted tissue therapy led to necrotic cells, inducing profound inflammation and cytotoxic T lymphocyte (CTL) killing. It was observed that eHsp70 initiated the production of IL-6, TGF-beta and the highly inflammatory IL-17 and tumour rejection by antigen specific CTL (Daniels et al., 2004). Incidentally, Xinjing et al. reported that eHsp70 played an inhibitory role on the stimulation of inflammatory cytokines, expressed by the activation of signalling pathways such as MAPK and NF-kB. The study conducted observed that TNF-α activated the mentioned pathways therefore increasing the expression of IL-6, IL-8 and MCP-1, however Hsp70 was able to inhibit the activation of this pathway (Luo et al., 2008). The difference of role is yet to be understood, but one could assume that the difference comes about due to the type of cells releasing the HSPs and the cells the HSPs will bind to.

1.5 Intra and Extracellular HSPs and skeletal muscle

Skeletal muscles cells are able to express HSPs (commonly Hsp70), indicative of a cytoprotective mechanism. Intracellular Hsp70 have demonstrated anti-inflammatory properties in skeletal muscles cells, being cytoprotective through anti-apoptotic mechanisms, inhibiting gene expression and

regulating cell cycle progression (Bittencourt and Porto, 2015). Aside from the commonly known chaperone function of Hsp70, recent studies have found that it is able to inhibit nuclear NF-kB activation, implicating significant effects on immunity, inflammation and cell survival. The inhibition can be seen at different levels; For example, heat induced Hsp70 is able to directly bind to IkB kinase gamma, therefore inhibiting the expression of TNFα, consequently preventing apoptosis (Beere, 2005). Several studies have suggested that Hsp70 is a natural inhibitor of the Nf-kB pathway, as it was seen that Hsp70 was able to inhibit the effect of TNF-a induced phospholipase A2, the suppression of nitric oxide expression correlating with decreased expression Nf-kB (Homem de Bittencourt and Curi, 2001). Additionally, the inhibitory effect of Hsp70 on apoptosis is profound. Caspases initiate an apoptotic cascade by the intrinsic pathway, characterised by the release of mitochondrial pro-apoptotic factors into the cytosol. The inhibitory effect of Hsp70 on apoptosis is characterised by many intracellular pathways e.g., JNK, NF-kB and Akt, which are either directly or indirectly blocked by Hsp70 (Rossi et al., 2000). Hsp70 massively impacts skeletal muscle disuse and wasting, as NF-kB expression has been shown to increase 3-fold in prolonged periods of atrophy and 5-fold in aged-disuse muscle (Jackman et al., 2012). However, increasing the expression of Hsp70 allows for the reversal of muscle wasting; achieved by inhibiting NF-kB activity (Dodd et al., 2008).

HSPs have demonstrated their ability to interact with several signalling molecules, regulatory factors and kinases such as IKKb, Akt and JNK. The anti-apoptotic functionality of HSPs has shown to specifically target the JNK pathway (Meriin et al., 1999). A study conducted by Park et al. showed that preventing the induction of Hsp72 allowed for the complete activation of the JNK pathway, in a mouse embryonic fibroblast cell line treated with a mild heat shock (Park et al., 2001). Furthermore, Hsp72 has been shown to prevent the onset of diet-induced insulin resistance in skeletal muscle cells. It was found that daily intake of Lipoic acid induced Hsp72 expression, which prevented insulin resistance (Gupte et al., 2009). The possible mechanisms accounting for the inhibitory effect of HSPs on the JNK pathway, are still under research. One possible mechanism is the direct binding of HSP, preventing the upstream activation of SAPK/Erk kinase 1 and MAP kinase kinase 7. Direct binding would also suggest inhibition of enzymatic activity of JNK or inhibiting the interaction between its substrate c-jun (Park et al., 2001). Another possible mechanism for the inhibition of JNK pathway is activating the upstream phosphates such MAP kinase phosphatase or dual leucine zipper-bearing kinase (Meriin et al., 1999).

Hsp70 has received notable attention for its role in maintaining skeletal muscle integrity and mass, protecting against muscle damage and promoting muscle recovery and regeneration. Studies have

shown Hsp70 to have increased in the case of exercise (Morton et al., 2009), muscle injury (Senf et al., 2013) and has been shown to promote muscle recovery and regeneration followed by exercise and muscle injury. Interestingly, it was found that Hsp70 also reduces during periods of muscle inactivity, therefore suggesting Hsp70 plays a significant role in muscle plasticity**.** McArdle et al., (2003) were the first to correlate Hsp70 and muscle plasticity, and this was achieved by using two groups of mice: wild type (WT) and Hsp70 transgenic mice (Tg). The findings of this study showed that Tg mice displayed significantly less muscle damage and were able to recover much more quickly in comparison to the WT; thus, suggesting Hsp70 is protecting against muscle damage and increasing muscle recovery. A similar study conducted by Miyabara et al., (2012) also displayed similar observations in the Tg mice, demonstrating increased recovery of fibre cross sectional area and functionality following induced muscle atrophy.

In contrast to the aforementioned studies investigating the significance of Hsp70 for muscle recovery, growth and prevention of muscle damage, another series of studies investigated the absence of Hsp70 and the resulting impact it would have on skeletal muscle cells; an meaningful investigation as the regeneration capabilities of skeletal muscle cells reduces as age increases, preventing muscle recovery and growth (Vasilaki et al., 2002)**.** Senf, (2013) investigated the effect of direct injury using cardotoxins, which induces muscle fibre necrosis, in mice lacking the Hsp70 gene. The investigators observed that the mice lacking the Hsp70 gene displayed delayed inflammation in response to induced injury, which later followed up with inflammation, muscle fibre necrosis and reduced regeneration of myofibers**.** The findings of this study provide concrete evidence in support of Hsp70's role in muscle recovery and regeneration, also highlighting Hsp70 expression decline with age. Taking into consideration the vast amount of studies investigating the role of intracellular HSPs and their findings, there is significant evidence to prove that HSPs have a profound role in maintaining muscle mass and integrity, inducing muscle regrowth and preventing muscle mass damage. However, most of what is known relates to intracellular HSPs, and relatively little is known about their role as extracellular HSPs in skeletal muscles.

As previously mentioned, upon release into extracellular space, HSPs can bind to a wide range of receptors and incite several signalling pathways, depending on the type of cell they bind to. Extracellular HSPs have been seen to have immunostimulatory functions when released into the extracellular space. Studies conducted upon heart, skin and liver tissue have demonstrated the recruitment of immune cells to the site of injury while also initiating pro-inflammatory processes. Senf et al. (2013) carried out a study which involved stimulating the release of Hsp70 into the extracellular

space, in mice lacking the Hsp70 gene. The investigators observed that in doing so they were able to induce a complete early immune infiltration into damaged muscle cells. Hsp70, commonly, is known to regulate the inflammatory response in skeletal muscles. Being an endogenous ligand for immune cell receptors means that it can stimulate macrophages, neutrophils and chemotaxis (Giraldo et al., 2009). Some investigators predict that Hsp70 is realised from necrotic cells, or through active secretion from compromised cells to act as a danger signalling molecule (Bausero et al., 2005). Evidence suggests eHSPs have a significant effect on skeletal muscles, however further investigation is required to properly understand the mechanism allowing eHSPS to have a potential protective role on skeletal muscle cells.

1.6 Aims, Objectives and Hypothesis:

1.6.1 Aim

Given the aforementioned gap in knowledge, this project aims to investigate the impact of eHSPs, particularly Hsp60 and Hsp70, on skeletal muscle, specifically examining their impact on muscle growth and metabolism.

1.6.2 Objectives

1. Analyse the viability of human skeletal muscle cells when treated with increasing concentrations of recombinant eHSPs
2. Investigate the effect of recombinant eHSPs on human skeletal muscle cells assessing mitochondrial activity
3. Investigate the effect of monocyte secretome (stimulated to secrete a pro-inflammatory profile) on muscle cell viability and mitochondrial activity

1.6.3 Hypothesis:

eHSPs can positively interact with skeletal muscle cells and modulate muscle growth and metabolism.

2.0 Materials and Methods

Details of the materials used in this thesis, can be seen in Table 2.1.

Table 2.1: Details of the materials used in this thesis.

Reagent	Manufacturer	Catalog no.
Dulbeccos modified eagles medium	Lonza	BE12-604
C2C12 murine myoblast cell line	ATCC	CRL-1772
Fetal Bovine Serum	Gibco	10438026
L-Glutamine	Lonza	BE17-605E
Gentamicin	Sigma-Aldrich	G1272
Human Recombinant Insulin	Sigma-Aldrich	91077C
Hepatocyte human growth factor	Gibco	PHG0321
Epidermal human growth factor	Gibco	RP-10914
Fibroblast human growth factor	Gibco	J67402.EXF
Dexamethasone	Sigma-Aldrich	D4092
Penicillin/Streptomycin	Lonza	09-757F
Trypsin EDTA	Lonza	BE17-161E
Myelomonocytic Cell Line	ATCC	CRL-1593.2
Rosewell Park Memorial Institute	Lonza	12-167Q
Trypan Blue	Gibco	15250061
Recombinant HSP 60	Bio-techne	AP-140-050
Recombinant HSP 70	Bio-techne	AP-100-050
TAK242	Bio-techne	6587/5
WST-8	Abcam	ab228554
Medium-199	Lonza	BE12-604

2.1 Cell Culture

The C2C12 murine myoblast cell line (ATCC, CRL-1772) was cultured in growth medium composed of 99% (v/v) Dulbecco's modified eagles medium, medium-199 supplemented (Lonza, BE12-604) with 20% (v/v) heat inactivated fetal bovine serum (Gibco, 10438026.), 1% (v/v) L-glutamine (Lonza, BE17-605E), 1% (v/v) penicillin/streptomycin (Lonza, 09-757F), 10ug/ml gentamicin (Sigma-Aldrich, G1272.), human recombinant 5ug/ml human insulin (Sigma-Aldrich, 91077C), 2.5 ng/ml hepatocyte human growth factor (Gibco, PHG0321), 5 ng/ml epidermal human growth factor (Gibco, RP-10914), 0.5 ng/ml fibroblast human growth factor (Gibco, J67402.EXF), 0.2ug/ml dexamethasone (Sigma-Aldrich,

D4092) and 25 ng/ml of fetal growth factors (Thermo Fisher, A4766801). Cells were incubated in a 5% CO_2 incubator until 80% confluence. Once C2C12 cells were 80% confluent, Trypsin EDTA (BE17-161E) was used to detach adherent cells from flask, allowing for sub-culture.

The U937, myelomonocytic cell line (ATCC, CRL-1593.2) was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Lonza, 12-167Q), supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were incubated in a 5% CO_2 incubator and regularly passaged to maintain a cell density of 1 X 10^5 and 2 X 10^6 viable cells/mL.

Prior to each experiment, cells were counted and assessed for cell viability using the Trypan Blue Exclusion method. Briefly, a 1:1 ratio of cell culture: trypan blue (Gibco, 15250061) was added to a haemocytometer and inserted into the TC10 automated cell count analyser (BioRad). Experiments were only performed when cell viability was >95%. Cell counts were adjusted accordingly prior to experiments by diluting in relevant medium. Using the equation $C_1 \times V_1 = C_2 \times V_2$, in the rearranged format of $V_1 = (C_2 \times V_2) / C_1$, the amount of solution containing cells required to achieve the desired cell density was calculated.

2.2 Recombinant HSP treatments

C2C12 cells were seeded at a density of 12 x10^4/ well in 100µl of growth media in 96 well plates. Following seeding, cells were left to pre-incubate for 24 hours in a 5% CO_2 incubator for 24 hours to adhere to the plate. HSPs were reconstituted in 38µl of DMSO, following this 3.86µl of HSP solution was added to 1ml of DMEM; allowing for a stock solution of 5mg/ml. Stock solution was further diluted using DMEM to achieve desired concentrations for treatment. The C2C12 cells were then treated with 10µl of the corresponding concentrations (5ng/ml, 10ng/ml, 25ng/ml, 50ng/ml) of recombinant HSP (Bio-Techne, AP-140-050, AP-100-050) or TLR4 inhibitor TAK242 (Bio-techne, 6587/5) and left to incubate for 24 hours. A media control was used by adding 10ul of DMEM to cells. This was used for all experiments.

2.3 Isolation of U937 conditioned media

Following adjustment of cell counts across T25 flasks, U937 cells were resuspended in DMEM media, since RPMI media was shown to effect viability of C2C12 cells in downstream experiments. Cells were treated in flasks accordingly. U937 cells were treated with 100ng/ml LPS made up in DMEM (Lonza, BE12-604) for either 1,2 4 or 24 hours at 37°C. After the corresponding treatment time, cells were washed and resuspended in fresh DMEM. For the controls, one flask remained untreated and

incubated at 37°C, while another, the 'heat shock control', was incubated at 42°C for 1 hour followed by a 23-hour recovery at 37°C. After the recovery period had elapsed, cells were centrifuged at 500*g* for 5 minutes and the supernatant was collected. Conditioned media was then used to treat C2C12 cells in attempt to mimic the environment similar to an ICU patient.

2.4 Cell viability (WST-8 assay)

Cell viability was assessed using the WST-8 assay. This is a tetrazolium salt, colorimetric assay which produces a water-soluble, orange-coloured formazan dye as its end product. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product. The amount of formazan produced is directly proportional to the number of living cells. This assay was chosen for the present work since, the detection sensitivity is significantly higher than assays using the other tetrazolium salts such as MTT, XTT, MTS or WST-1. WST-8 assay can also be indicative of increased metabolism to prevent cellular death as a result of stress. Enzymes such as SIRT3 and IDH2 are overproduced in attempt to counteract the effects of oxidative stress and may lead to the increased production of NADPH, the bioreducing molecule to produce formazan dye (Smolková and Ježek, 2012).

C2C12 cells were seeded at a density of 12 $x10^4$/ well in 100µl of growth media in 96 well plates. Following seeding, cells were left to pre-incubate for 24 hours in a 5% CO_2 incubator for 24 hours to adhere to the plate.

For U937 cells, these were seeded at 3 X 10^5 cells/well in 100µl of RPMI growth media and treated immediately. The C2C12 or U937 cells were treated with 10µl of the corresponding treatment (recombinant HSP, TAK242 or U937-conditioned media) and left to incubate for 24 hours. The C2C12 cells were then treated with 10µl of WST-8 solution (Abcam, ab228554) and left to incubate for 1 hour before reading the plate in an absorbance reader at the recommended 450nm. A dead cell control (DCC) was included in the WST-8 assay; C2C12 or U937 cells were treated with 70% Ethanol for 24 hours.

2.5 Extracellular Flux Analyser to assess mitochondrial oxygen consumption

Oxygen consumption rate (OCR) of C2C12 cells was measured using the XFp extracellular flux analyser (Seahorse Bioscience, Agilent technologies). OCR provides a direct indicator of mitochondrial respiration. However, ATP production can also occur independently of oxygen, via glycolysis (conversion of glucose to lactate). Measurement of the extra-cellular acidification rate (ECAR) gives a direct indicator of lactate production, since the lactate in the cell culture medium causes acidification of the medium, again providing an indication of mitochondrial respiration.

Cells were seeded at a concentration of $5x10^4$/well in 100µl of growth media in a XFp cell culture plate and left to incubate for 24 hours in a 5% CO_2 incubator. The C2C12 cells were then treated with doses of corresponding treatment and left to incubate for 24 hours in a 5% CO_2 incubator. The sensor cartridge was hydrated using XFp calibrate and left to incubate in a non- CO_2 incubator overnight. Growth media was substituted with Seahorse XF base medium (Agilent, 102353-100) supplemented with 10mM glucose (Sigma, D9434), 2mM L-glutamine (Sigma, G8540) and 1mM pyruvate (Thermo Fisher, 11360070). In order to determine proton leak, maximal respiration and non-mitochondrial OCRs, Oligomycin (20µL), FCCP (22µL) and Rotenone (25µL) were loaded onto a XFp cartridge plate. OCR and extracellular acidification were automated by Wave software (Agilent technologies). Collected data was expressed as pmol of oxygen/min and normalised to total cellular protein according to the Bradford assay (Olson BJ, 2016).

2.6 Data Handling and Statistics

All data was analysed using GraphPad PRISM 9. In WST-8 assays, data were normalised the experimental controls; The dead cell control (DCC represented 0% cell survival, while the media control represented 100% cell survival. The effect of treatments (recombinant HSPs, TAK242 or U937 conditioned medium) on C2C12 viability or mitochondrial respiration was tested using the one-way ANOVA with Dunnett's post hoc test, in addition with Kolmogorov-Smirnov to test for normality, using $p<0.05$ as the threshold for significance. Significant differences from the control are indicated on the corresponding figures.

3.0 Results

3.1. The effect of extracellular (recombinant) Hsp70 and Hsp60, and the TLR4 inhibitor, TAK242, on myoblast viability.

3.1.1 Effect of extracellular HSPs on myoblast viability

The first step in this project was to determine the effect of extracellular Hsp70 on the viability of the myoblasts, to ascertain whether this circulating inflammatory mediator could be involved in muscle cell degradation.

C2C12 cells were treated with varying (physiologically relevant) concentrations of recombinant HSP70 for 24 hours before assessment of cell viability using the WST-8 assay (Figure 3.1). The results show an overall trend of decreasing viability as the concentration of Hsp70 is increased. Statistical analysis revealed a significant decrease in cell survival at concentrations of 10ng/ml, 25ng/ml and 50ng/ml. Data are presented as a mean +/- SD * p 0.05 against control (N=6)

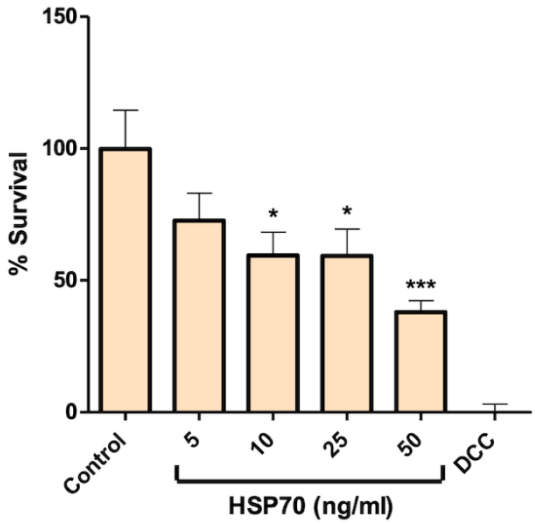


*Figure 3.1: The effect of recombinant Hsp70 on cell viability of C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of HSP70. DCC represents the dead-cell control. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. Significant differences from the control are indicated with *. N=6 for each treatment group.*

Likewise, the effect of extracellular Hsp60 on the viability of the myoblasts was studied. Again, C2C12 cells were treated with varying (physiologically relevant) concentrations of recombinant Hsp60 for 24 hours before assessment of cell viability using the WST-8 assay (Figure 3.2). The results show an overall decrease in cell survival with all treatments; however, this was only seen to be statistically significant at a concentration of 5ng/ml. Data are presented as a mean +/- SD * p 0.05 against control (N=6)

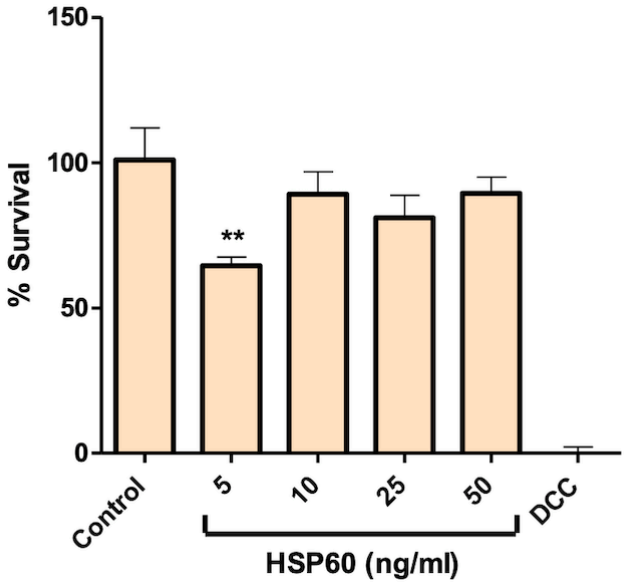


*Figure 3.2: The effect of recombinant Hsp60 on cell viability of C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp60. DCC represents the dead-cell control. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using $p<0.05$ as the threshold for significance. Significant differences from the control are indicated with *. N=6 for each treatment group.*

3.2. The effect of extracellular (recombinant) Hsp70 and Hsp60, and TAK242 on myoblast respiration

The effects of extracellular Hsp70 on several aspects of cellular respiration, (1) non-mitochondrial respiration, (2) basal respiration, (3) maximal respiration, (4) proton leak, (5) ATP production, (6) spare respiratory capacity and (8) coupling efficiency, was studied using the Seahorse XF Analyser. Non-mitochondrial respiration is a measurement of the oxygen consumed by other cellular processes outside of the mitochondria such as NADPH oxidases, which consume a vast amount of oxygen in macrophages. Rotenone plus antimycin A is able inhibit the mitochondrial electron transport chain enabling the visualisation of mon-mitochondrial oxygen consumption. Non-mitochondrial respiration was seen to increase when treated with 5ng/ml Hsp70, although this was not statistically significant (Figure 3.4A), and despite the initial observation of an increase following 10ng/ml Hsp60 (Figure 3.4B), this was also statistically insignificant. Overall, none of the concentrations of Hsp70 or Hsp60 tested had a significant effect on non-mitochondrial respiration (Figure 3.4). Data are presented as a mean +/- SD * p 0.05 against control (N=4)

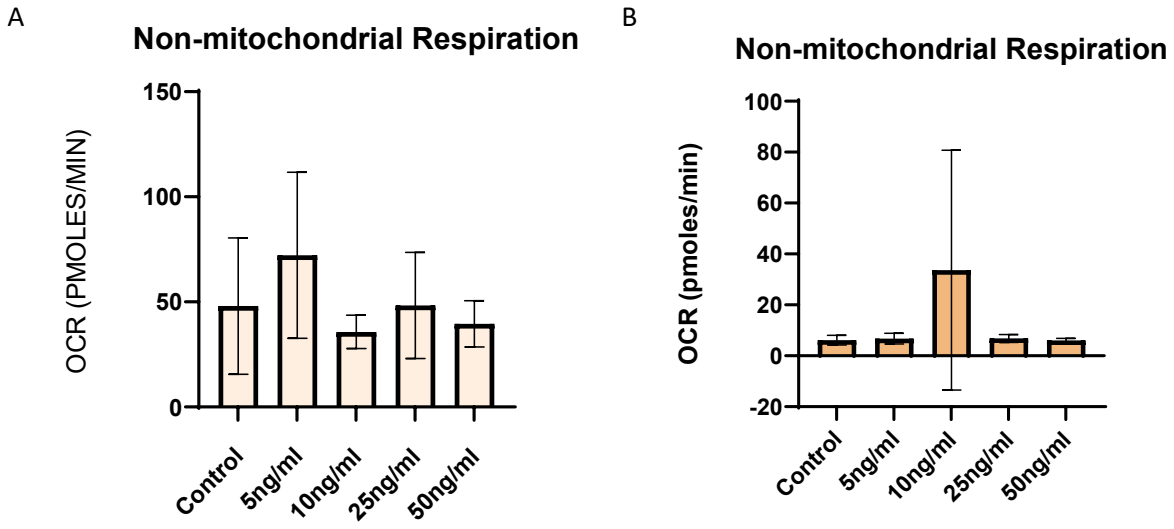
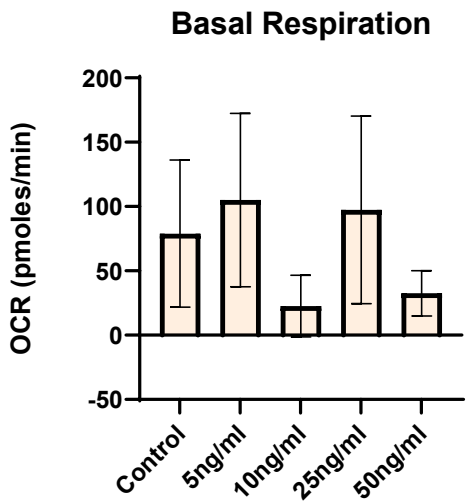


Figure 3.4: The effect of recombinant (A) Hsp70 and (B) Hsp60 on non-mitochondrial respiration in C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp70 or Hsp60. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

Basal respiration represents the energetic demand of cells under basal conditions. A change in basal respiration rate indicates there is some change taking place. However further investigations are required to pinpoint the nature and location of the change. Overall, no significant differences were observed in basal respiration at any of the Hsp70 or Hsp60 concentrations tested (Figure 3.5), despite a general trend of decreased activity at 10 and 50ng/ml Hsp70 (Figure 3.5A) and a general trend of increased activity at 10ng/ml Hsp60 (Figure 3.5B). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

A



B

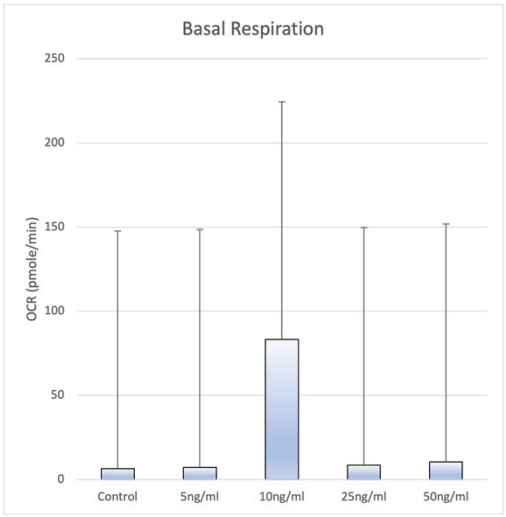


Figure 3.5: The effect of recombinant (A) Hsp70 and (B) Hsp60 on basal respiration in C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp70 or Hsp60. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using $p<0.05$ as the threshold for significance. N=4 for each treatment group.

Maximal respiration is the maximum capacity the electron transport chain can achieve, this can be achieved by the addition of an uncoupler, such as FCCP. The increased demand in energy does not increase substrate supply, causing a lack of cytoplasmic ATP to maintain catabolic pathways. Increased maximal respiration is indicative of mitochondrial dysfunction. Again, none of the Hsp70 or Hsp60 concentrations tested resulted in a significant change in maximal respiration (Figure 3.6). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

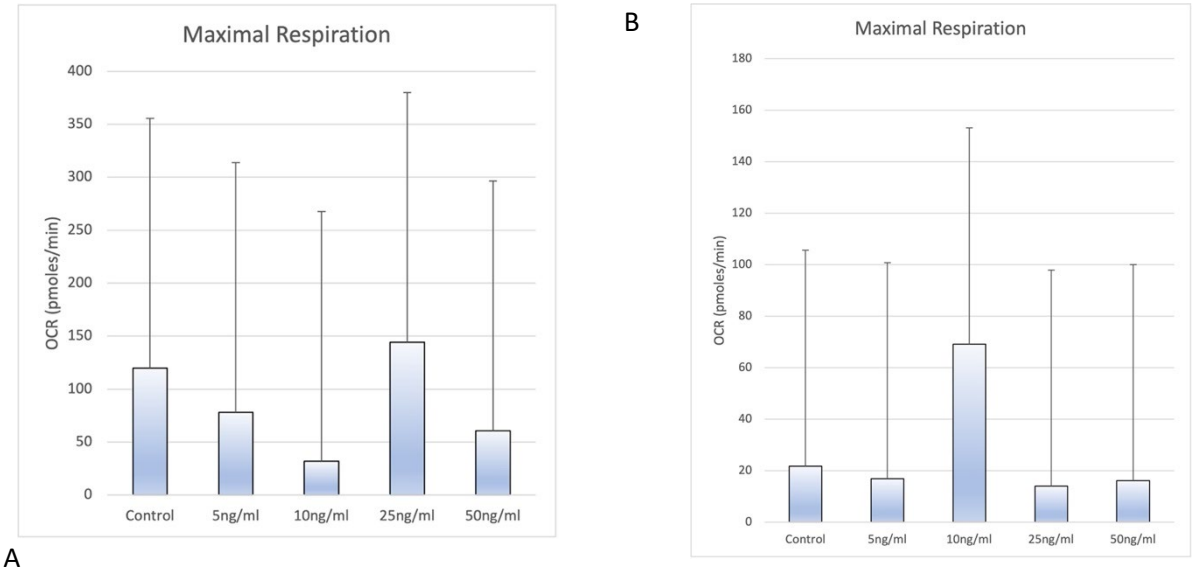


Figure 3.6: The effect of recombinant (A) Hsp70 and (B) Hsp60 on maximal respiration in C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp70 or Hsp60. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

Proton gradient on the inner mitochondrial membrane ensures respiratory oxygen to be used in ADP phosphorylation/ATP generation, hence why the proton gradient is crucial for ATP production. Proton leak takes place when protons migrate independently of ATP synthase. Proton leak can be mimicked using oligomycin, an ATP synthase. Despite a seemingly noteworthy rise in proton leak following treatment with 5ng/ml Hsp70 (Figure 3.7A) and 10ng/ml Hsp60 (Figure 3.7B), statistical analysis revealed that neither Hsp70 nor Hsp60 do not affect proton leak in C2C12 cells at any of the concentrations tested (Figure 3.7). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

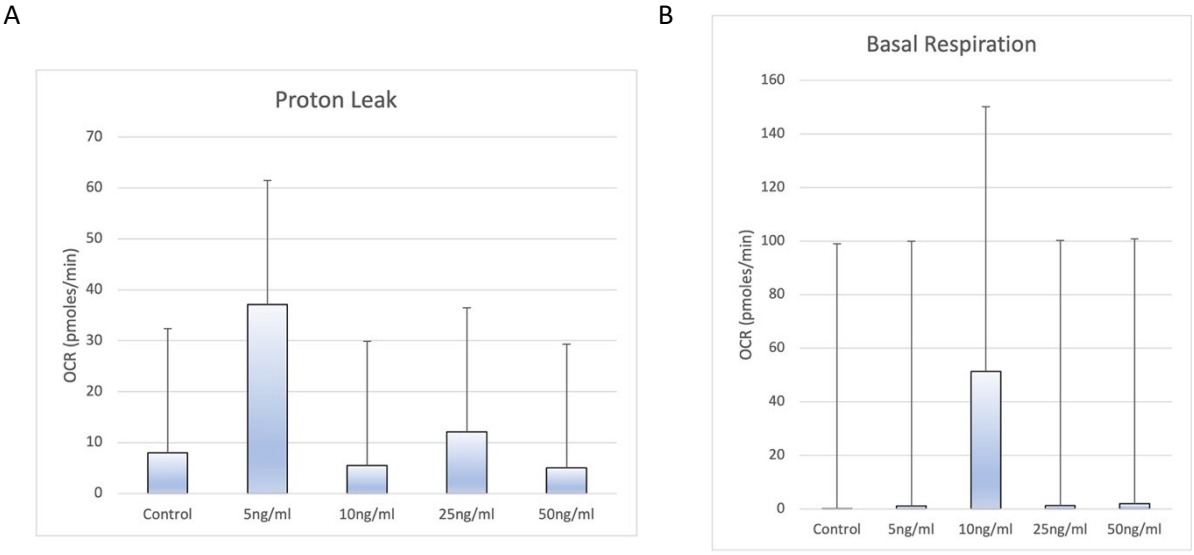
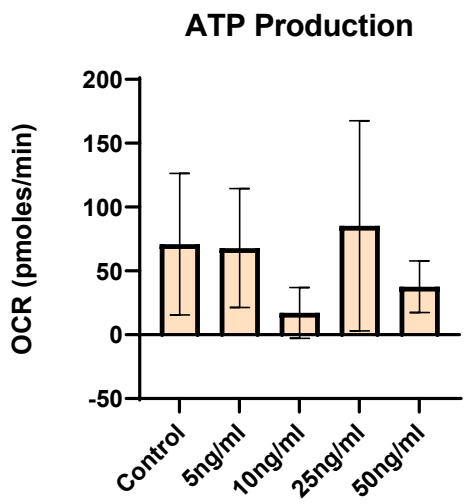


Figure 3.7: The effect of recombinant (A) Hsp70 and (B) Hsp60 on proton leak in C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp70 or Hsp60. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using $p<0.05$ as the threshold for significance. N=4 for each treatment group

ATP is an immediate power source for cell processes. During the exergonic process of electron transport, an electrochemical gradient enables protons to move back into the inner mitochondrial membrane, resulting in the production of ATP. In healthy cells, ATP production is unaffected but is reduced during mitochondrial dysfunction. None of the concentrations of Hsp70 or Hsp60 tested resulted in a statistically significant change in ATP production (Figure 3.8). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

A



B

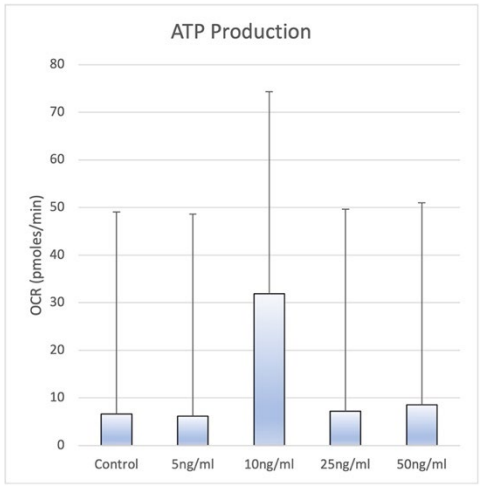


Figure 3.8: The effect of recombinant (A) Hsp70 and (B) Hsp60 on ATP production in C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp70 or Hsp60. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group

Spare respiratory capacity (SRC) is how well the supply of substrate and electron transport can respond to an increase in energy demand. Reduced SRC is indicative of mitochondrial dysfunction which may otherwise not be visible under basal conditions. SRC remained similar to control across all concentrations of both Hsp70 and Hsp60 tested (Figure 3.9). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

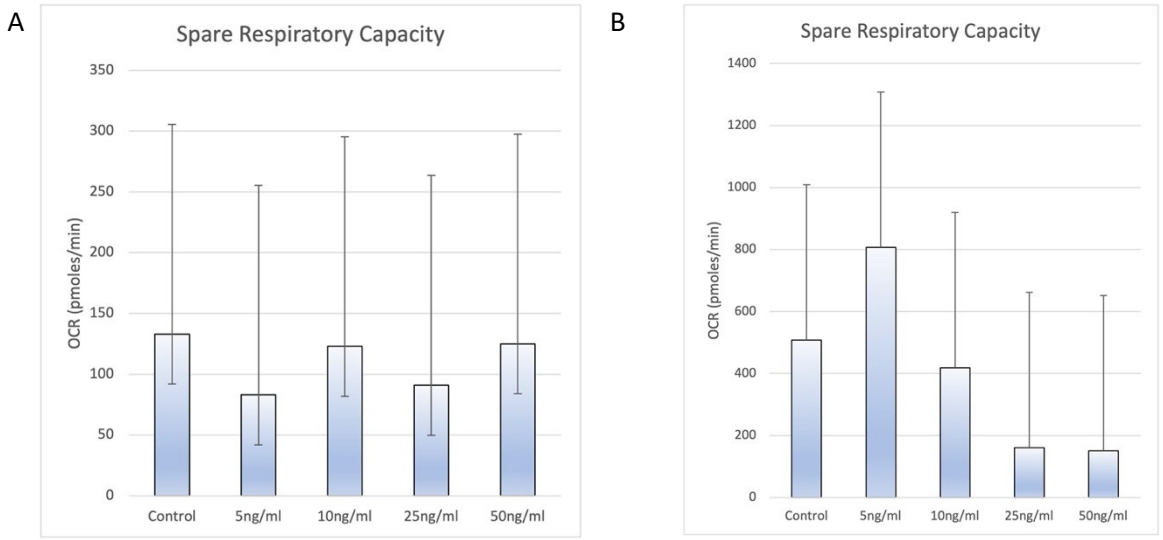
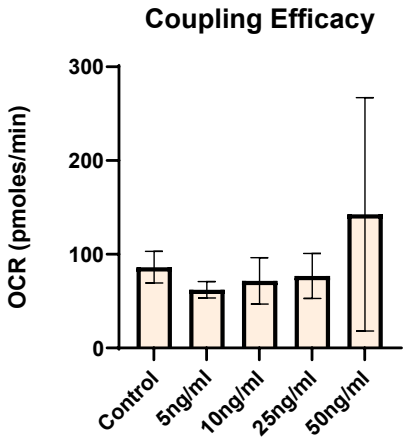


Figure 3.9: The effect of recombinant (A) HSP70 and (B) Hsp60 on spare respiratory capacity in C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp70 or Hsp60. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using $p<0.05$ as the threshold for significance. N=4 for each treatment group.

Coupling efficiency is defined by how well two substrates bind together, in the case of the mitochondrion it would be substrate oxygen and ATP generation. Coupling efficiency is greatly impacted by proton leak, therefore making it a good marker of mitochondrial dysfunction. The coupling efficiency is seen to remain statistically unchanged in the presence of increasing concentrations of Hsp70 (Figure 3.10A) and Hsp60 (Figure 3.10B). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

A



B

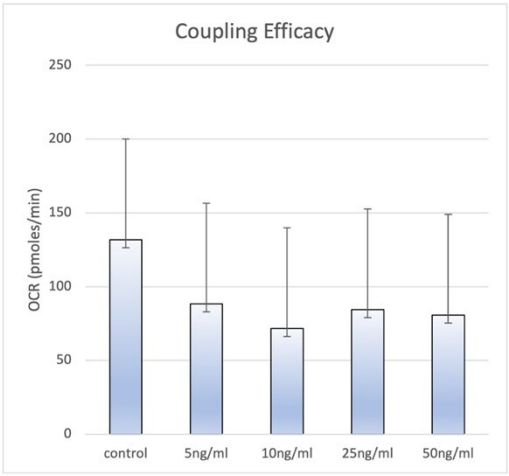


Figure 3.10: The effect of recombinant (A) HSP70 and (B) Hsp60 on coupling efficacy in C2C12 myoblasts. C2C12 cells were treated for 24 hours with (or without) varying doses of Hsp70 and Hsp60. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using $p<0.05$ as the threshold for significance. N=4 for each treatment group.

3.2.1 Effect of TAK242 on cellular respiration

As previously discussed, eHsp70 and eHsp60 are known to bind TLR-4. Therefore, the effect on respiration, of blocking TLR4 function using the TLR-4 specific inhibitor, TAT242, in the presence of the eHSPs was studied. TAK242 alone appeared to have an effect on mitochondrial respiration, but this was found to be statistically insignificant. Treating the cells with this inhibitor alongside the HSPs had no impact on non-mitochondrial respiration (Figure 3.11). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

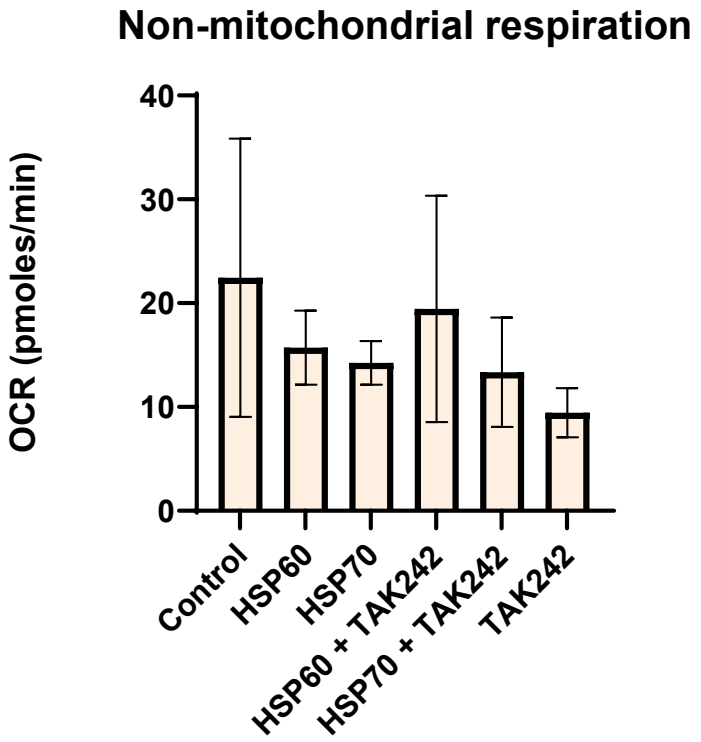


Figure 3.11: The effect of TAK242 alone, or in combination with Hsp70 or Hsp60 on non-mitochondrial respiration in C2C12 myoblasts. C2C12 cells were treated for 24 hours with TAK242 (xng/ml) with or without Hsp70 (50ng/ml) or Hsp60 (50ng/ml). Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

Basal respiration was seen to increase in comparison to control with all treatments, however blocking TLR-4 with TAK242 had no effect on how the cells behaved in response to eHsp70 or eHsp60 (Figure 3.12). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

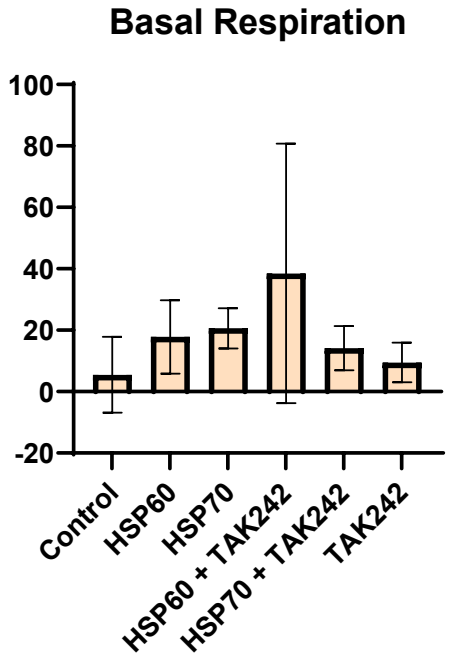


Figure 3.12: The effect of TAK242 alone, or in combination with Hsp70 or Hsp60 on basal respiration in C2C12 myoblasts. C2C12 cells were treated for 24 hours with TAK242 (ng/ml) with or without Hsp70 (50ng/ml) or Hsp60 (50ng/ml). Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

Likewise, although there appeared to be an increase in maximal respiration in some of the treatment groups, statistical significance was not observed in any of the data sets (Figure 3.13). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

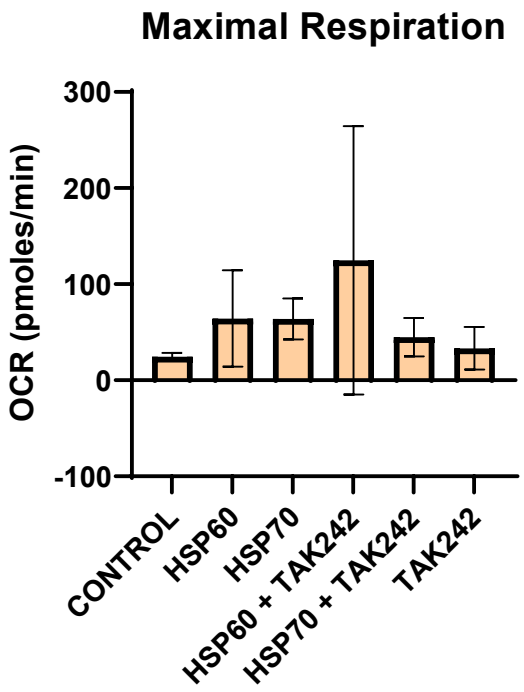


Figure 3.13: The effect of TAK242 alone, or in combination with Hsp70 or Hsp60 on maximal respiration in C2C12 myoblasts. C2C12 cells were treated for 24 hours with TAK242 (ng/ml) with or without Hsp70 (50ng/ml) or Hsp60 (50ng/ml). Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

Proton leak was seen to increase when treated with Hsp60, 70, Hsp60 and the HSPs in combination with TAK242. Yet only a statistically significant increase was seen with HSP60 + TAK242. Data are presented as a mean +/- SD * p 0.05 against control (N=4).

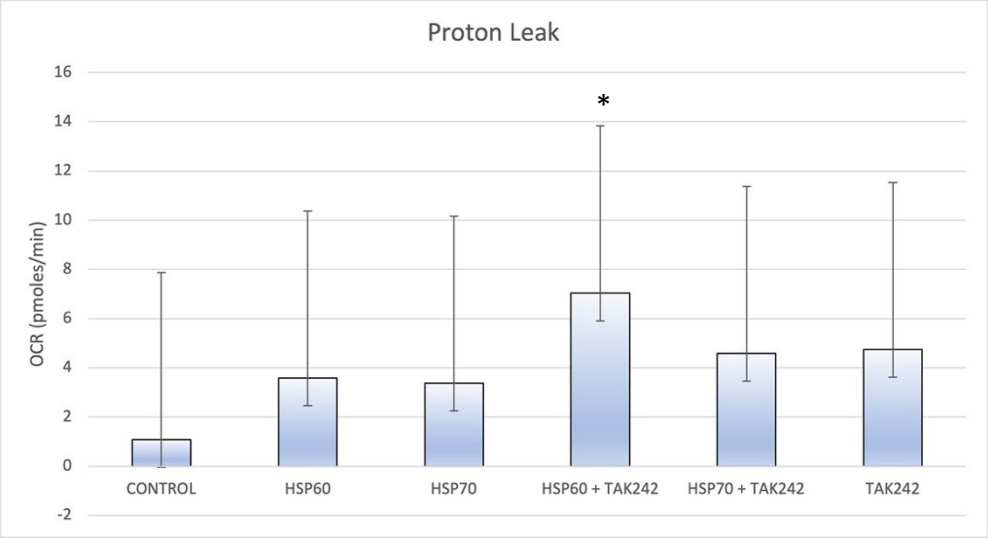


Figure 3.14: The effect of TAK242 alone, or in combination with Hsp70 or Hsp60 on proton leak in C2C12 myoblasts. C2C12 cells were treated for 24 hours with TAK242 (ng/ml) with or without Hsp70 (50ng/ml) or Hsp60 (50ng/ml). Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group

.

ATP production remained when cells were treated with eHsp70 in Hsp60 in the presence of TAK242 (Figure 3.15). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

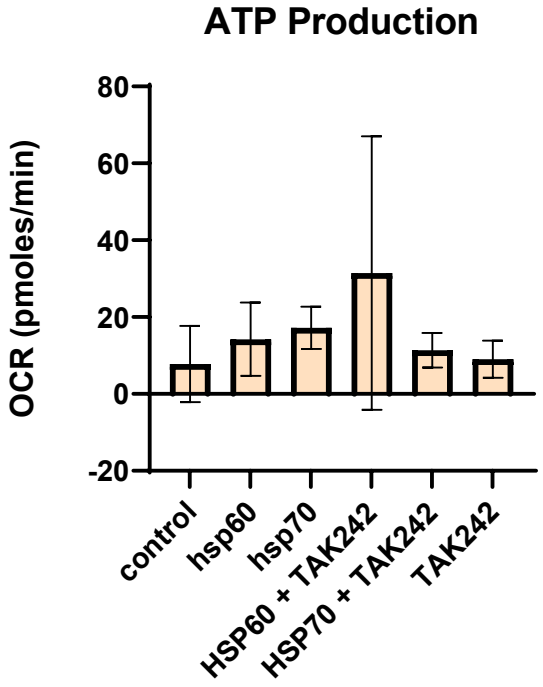


Figure 3.15: The effect of TAK242 alone, or in combination with Hsp70 or Hsp60 on ATP production in C2C12 myoblasts. C2C12 cells were treated for 24 hours with TAK242 (ng/ml) with or without Hsp70 (50ng/ml) or Hsp60 (50ng/ml). Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

.

Spare respiratory capacity was seen to increase with each treatment, a small statistically insignificant increase was seen when treated with HSP70 + TAK242 and TAK242, a greater statistically insignificant increase was seen when treated with HSP60, HSP70 and HSP60 + TAK242. Data are presented as a mean +/- SD * p 0.05 against control (N=4).

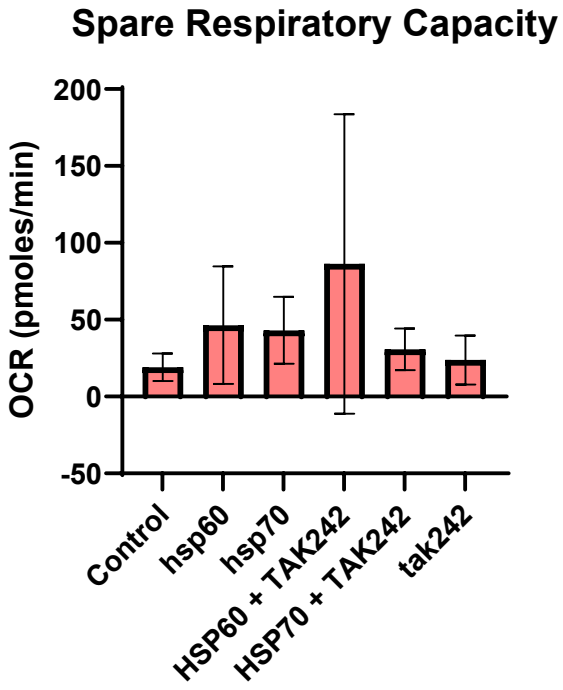


Figure 3.16: The effect of TAK242 alone, or in combination with Hsp70 or Hsp60 on spare respiratory capacity in C2C12 myoblasts. C2C12 cells were treated for 24 hours with TAK242 (ng/ml) with or without Hsp70 (50ng/ml) or Hsp60 (50ng/ml). Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

The addition of TAK242 to Hsp70 and Hsp60 treatments did not affect coupling efficacy at the concentrations tested (Figure 3.17). Data are presented as a mean +/- SD * p 0.05 against control (N=4).

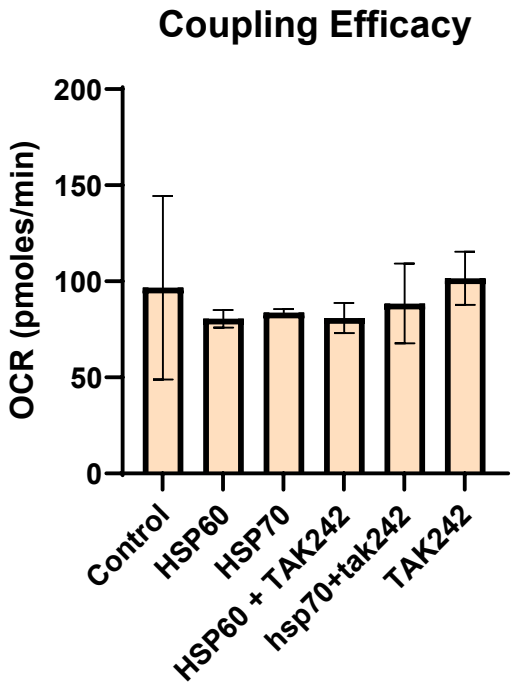


Figure 3.17: The effect of TAK242 alone, or in combination with Hsp70 or Hsp60 on coupling efficiency in C2C12 myoblasts. C2C12 cells were treated for 24 hours with TAK242 (ng/ml) with or without Hsp70 (50ng/ml) or Hsp60 (50ng/ml). Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=4 for each treatment group.

3.3. The effect of U937-conditioned medium on myoblast viability

In-vivo, monocytes are well-known major producers of pro-inflammtory cytokines during the cytokine storm, and the release of extracellular HSPs. To determine whether the monocyte 'secretome' as a source of native HSPs had any effect on muscle cell dysfunction, the U937 monocytic cell line was treated with Lipopolysaccharide to stimulate cytokine and HSP release, and the resutlant supernatant was collected before being applied to C2C12 cells. The results show a statistically significant decrease when C2C12 cells were treated with conditioned media from the LPS-stimulated U937s or LPS directly. As can be seen in Figure 3.18, U937 conditioned media had a significant effect on the viability of the C2C12 cells after 24 hours of treatment, and this effect was similar to that induced when treating the C2C12 cells directly with LPS. However, RPMI treatment alone also affected the viability of the C2C12 cells cultured in that media for 24 hours, and so from this, it was determined that any future experiments involving U937 conditioned media, needed to be conducted in DMEM. Data are presented as a mean +/- SD * p 0.05 against control (N=6)

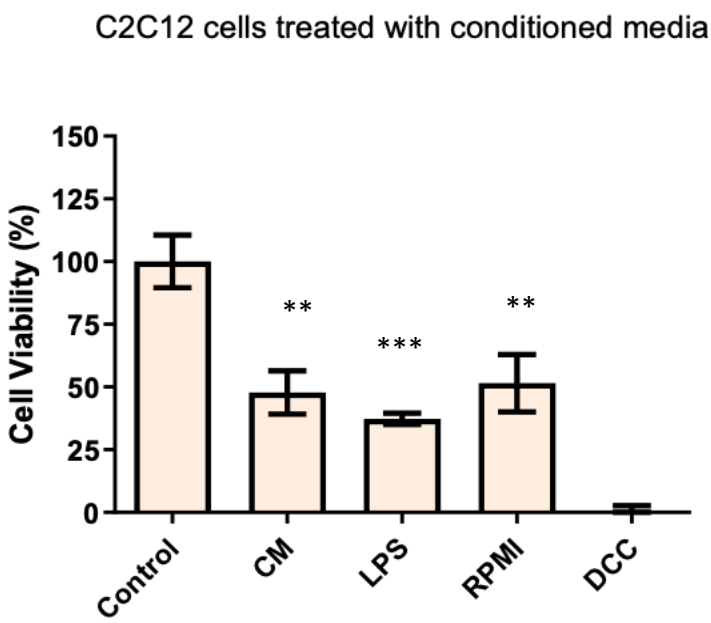


*Figure 3.18: The effect of U937 conditioned media (CM) or direct treatment of LPS (1000ng/ml for 1 hour) on the viability of C2C12 myoblasts. C2C12 cells were treated for 24 hours. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=6 for each treatment group. * Indicates significance.*

Next, a time-course experiment was set-up to investigate whether varying the duration of LPS-treatment on the U937s before collection of the conditioned media (in DMEM, not RPMI), had any downstream effect on the viability of the C2C12 cells. As seen in Figure 3.19, U937s treated for just 1 and 2 hours with LPS, produced conditioned media that had a significant effect in reducing C2C12 cell viability, in comparison with the control. Interestingly, this was not the case with treatments of longer duration (4 hours and 24 hours). Data are presented as a mean +/- SD * p 0.05 against control (N=6).

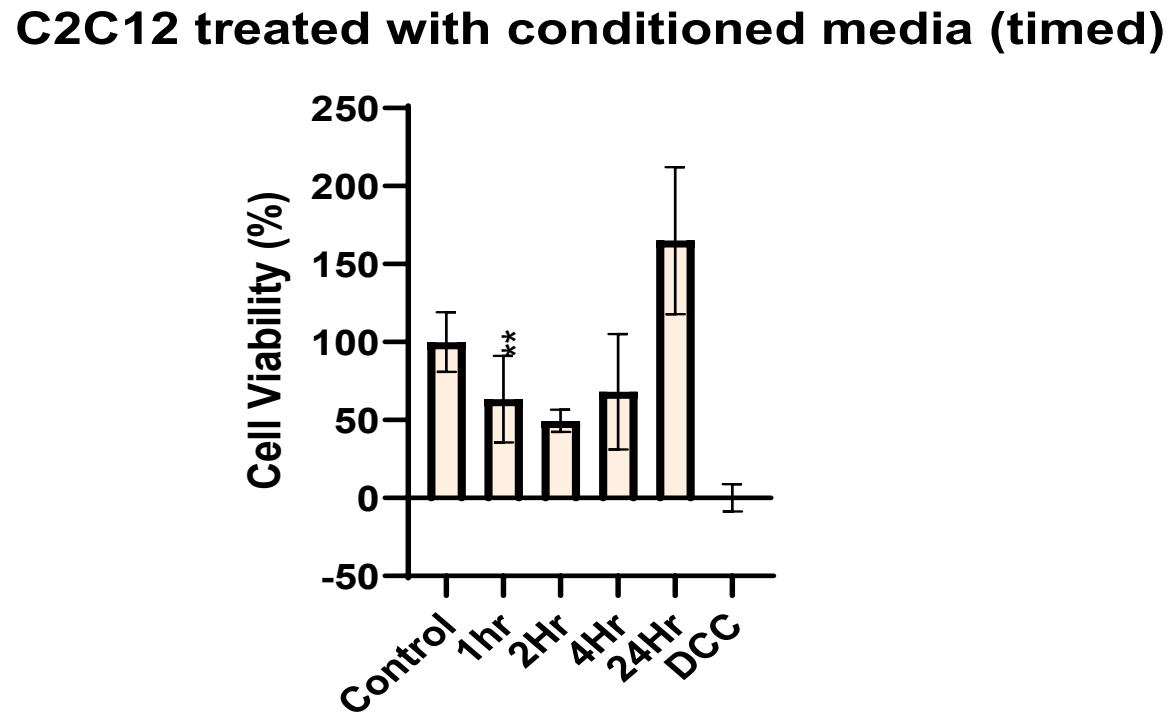


Figure 3.19: The effect of U937 conditioned media (CM), media on the viability of C2C12 myoblasts. U937 cells were treated for the indicated times with LPS (1000 ng/ml) before the cells were washed and left to 'recover' for 24 hours. The conditioned media was then collected and applied to C2C12 cells for 24 hours. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=6 for each treatment group.

3.3.1: Effect of U937 conditioned media on Myoblast respiration

The effects of LPS-stimulated U937 conditioned media on C2C12 cellular respiration was studied. U937s were stimulated for varying times (as indicated) before being allowed to recover for 24 hours in LPS-free media. The conditioned media (DMEM not RPMI) was then collected and applied to C2C12 cells for a further 24 hours.

In contrast to the insignificant effects observed by the extracellular recombinant HSPs, non-mitochondrial respiration was seen to increase in C2C12 cells treated with LPS-stimulated U937 conditioned media at all time points tested. Data are presented as a mean +/- SD * p 0.05 against control (N=8).

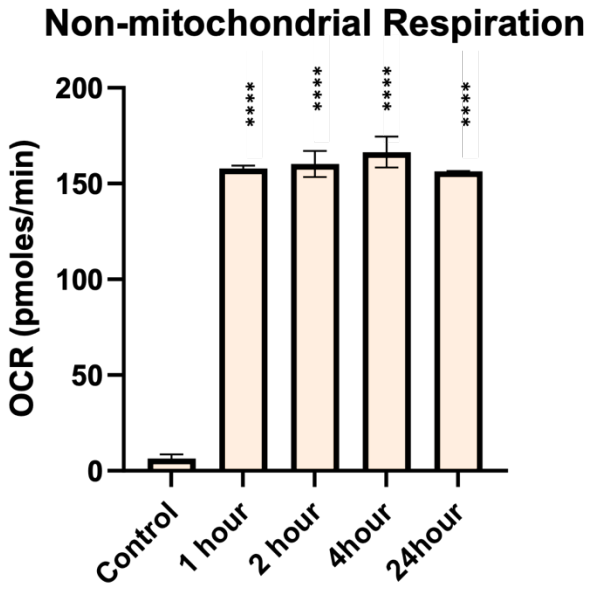


*Figure 3.20: The effect of U937-conditioned media, induced by LPS treatments (1000ng/ml) for 1hr, 2hr, 4hr and 24hr, on the non-mitochondrial respiration of C2C12 myoblasts. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=8 for each treatment group. **** indicates significance.*

In the same set of experiments, basal respiration was seen to increase across all treatment periods apart from the 24 hour treatment group which showed basal respiration levels similar to those in untreated samples (Figure 3.21). Data are presented as a mean +/- SD * p 0.05 against control (N=8).

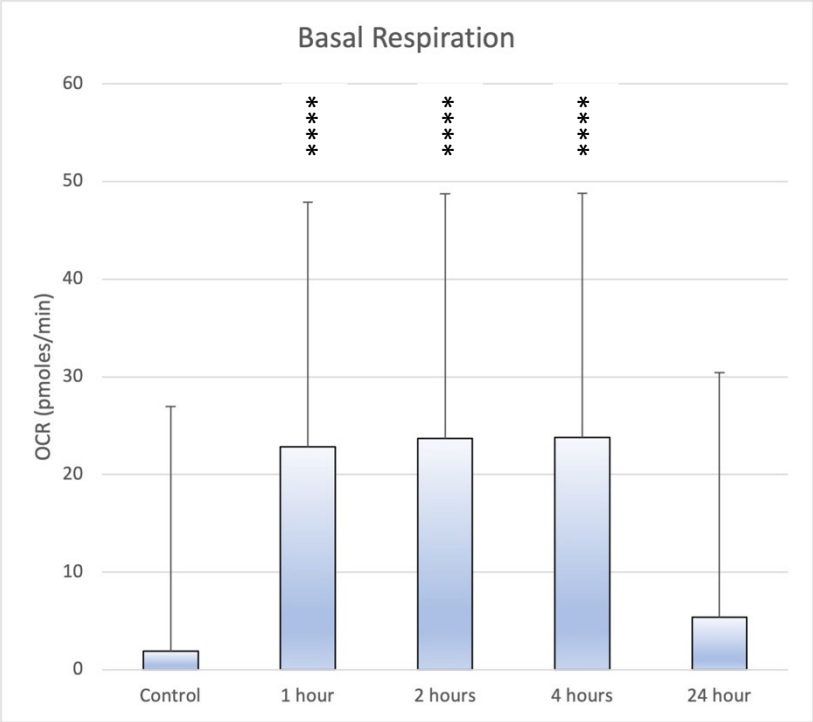


Figure 3.21: The effect of U937-conditioned media, induced by LPS treatments (1000ng/ml) for 1hr, 2hr, 4hr and 24hr, on the basal respiration of C2C12 myoblasts. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=8 for each treatment group.

Maximal respiration was seen to increase as the period of treatment increased, however dropped profoundly at 24hr, however there was no statistical significance. Data are presented as a mean +/- SD * p 0.05 against control (N=8)

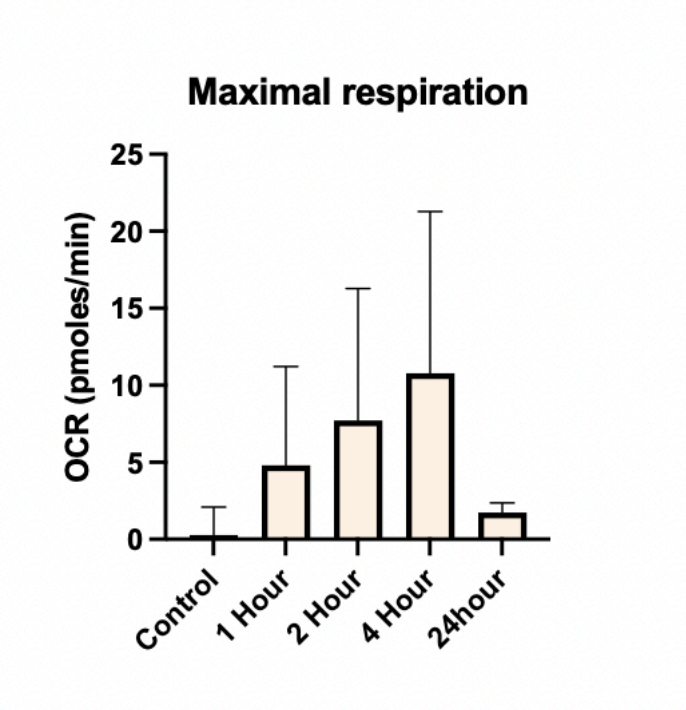


Figure 3.22: The effect of U937-conditioned media, induced by LPS treatments (1000ng/ml) for 1hr, 2hr, 4hr and 24hr, on the maximal respiration of C2C12 myoblasts. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=8 for each treatment group.

No significant difference in proton leak across treatment groups was observed, despite an apparent increasing trend with 1 hour and 2 hour treatments (Figure 3.23). Data are presented as a mean +/- SD * p 0.05 against control (N=8)

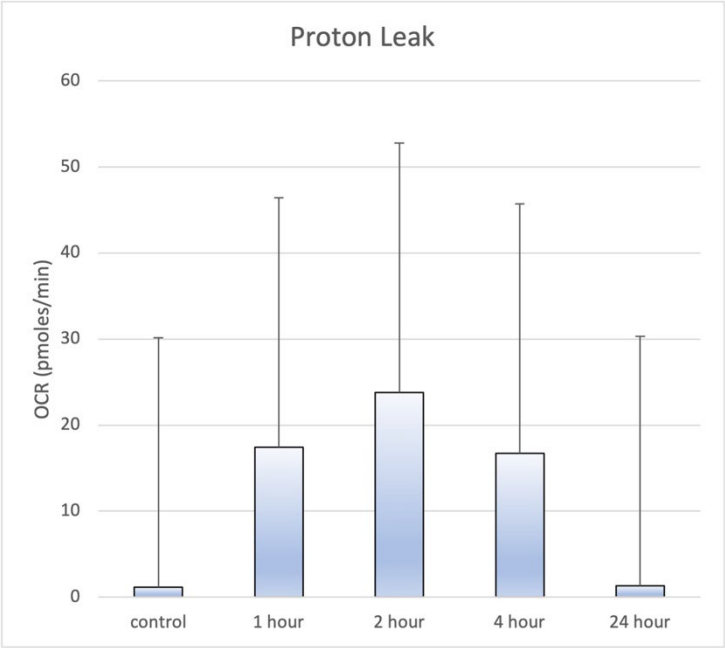


Figure 3.23: The effect of U937-conditioned media, induced by LPS treatments (1000ng/ml) for 1hr, 2hr, 4hr and 24hr, on the proton leak of C2C12 myoblasts. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=8 for each treatment group.

ATP production displayed a statistical increase when treated for 1 hour and 4 hours. ATP production was negligible when treated for 2 hours and 24 hours (Figure 3.24). Data are presented as a mean +/- SD * p 0.05 against control (N=8)

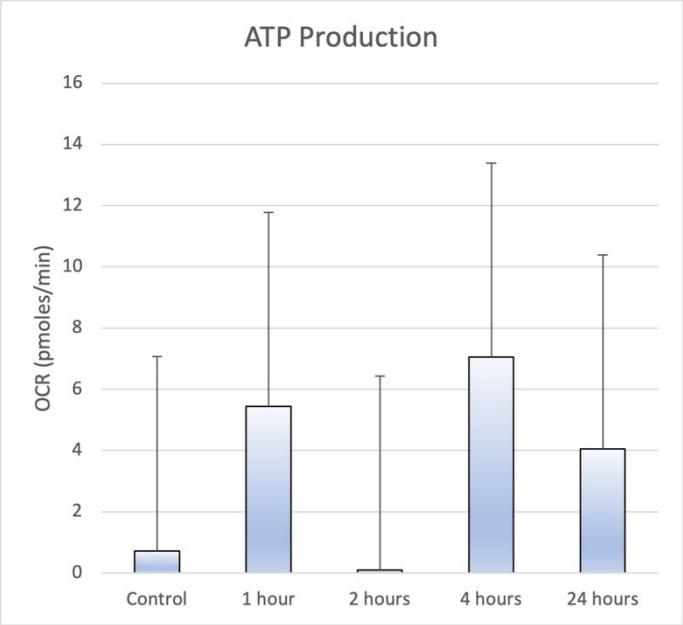


Figure 3.24: The effect of U937-conditioned media, induced by LPS treatments (1000ng/ml) for 1hr, 2hr, 4hr and 24hr, on the ATP Production of C2C12 myoblasts. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=8 for each treatment group.

Spare respiratory capacity was seen to decrease significantly after 1,2,4 and 24 hours of treatment when compared to untreated samples (Figure 3.25). Data are presented as a mean +/- SD * p 0.05 against control (N=8)

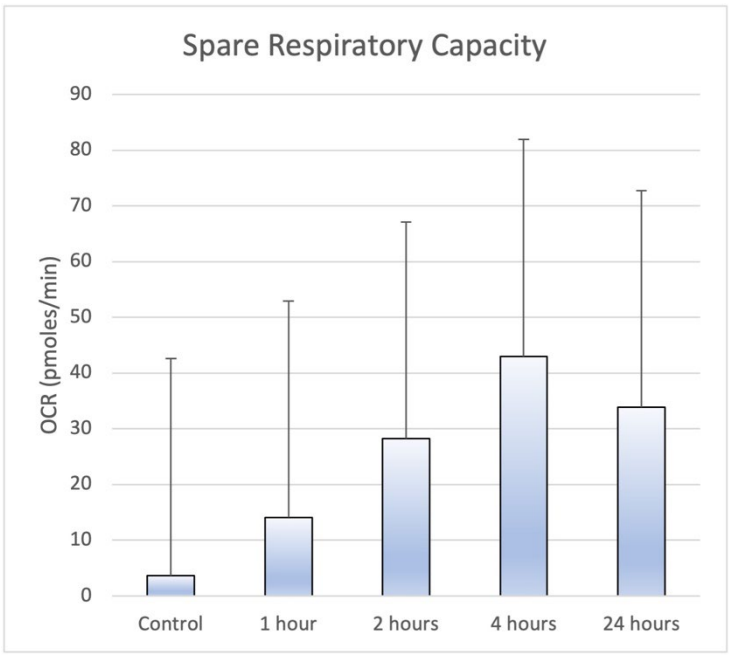


Figure 3.25: The effect of U937-conditioned media, induced by LPS treatments (1000ng/ml) for 1hr, 2hr, 4hr and 24hr, on the SRC of C2C12 myoblasts. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=8 for each treatment group.

Coupling Efficacy was not affected by the periods of treatment, despite the apparent increase seen after 1 and 24 hours of treatment (Figure 3.26). Data are presented as a mean +/- SD * p 0.05 against control (N=8).

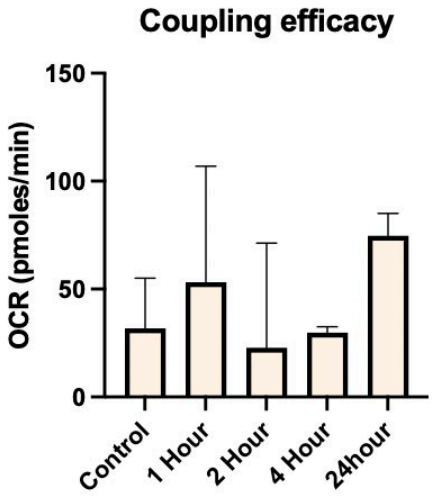


Figure 3.26: The effect of U937-conditioned media, induced by LPS treatments (1000ng/ml) for 1hr, 2hr, 4hr and 24hr, on the Coupling Efficacy of C2C12 myoblasts. Statistical analysis was performed using the one-way ANOVA with Dunnett's post hoc test, using p<0.05 as the threshold for significance. N=8 for each treatment group.

4.0 Discussion:

Objective:

HSPs have been investigated intracellularly for several decades, in many ways highlighting their key role as a molecular chaperone in the cell. Despite the significant role HSPs play intracellularly, there is lack of knowledge of their mechanisms once they are released into extracellular space. Current knowledge highlights their main role in immunity, assisting the activation of immune responses; however, while being mediators of immunity they can also induce pro-inflammatory cytokines which may produce free-radicals. NO, a free-radical, has been investigated to directly affect the functionality of the mitochondria by inducing respiratory inhibition (Brown, 2008). Therefore, this study investigated the role of eHsp60 and 70 in cell viability, mitochondrial function and whether there is cytotoxic or cytoprotective role.

4.1 Cell Viability:

4.1.1 Effect of recombinant HSPs on cell viability:

Cell viability is often used to visualise the effects of test compounds on cell proliferation or to highlight any cytotoxic effects induced by the test compound, which would cause cell death. A WST-8 assay was carried out on C2C12 cells treated with increasing doses of HSP60, it was found that the cell viability significantly reduced when treated with 5ng/ml alongside with the reduction of viability seen across all treatments. This cytotoxic effect of HSP60 could be hypothesised in two such ways; the first being its ability to stimulate inflammatory pathways, as discussed before HSPs can bind to several receptors such as TLR2 and TLR4, stimulating a plethora of inflammatory pathways inciting cell necrosis. Another potential mechanism is the lack of eHSPs stimulating immunostimulatory pathways, prevent a protective mechanism activating against cytotoxic molecules in the external environment. C2C12 cells treated with increased doses of HSP70 displayed a decrease in cell viability as the concentration of HSP70 increased, the most significant decrease was seen when treated with 50ng/ml. This falls in line with the findings of Daniels et al, who reported that an increase in eHSP70 expression incited cell necrosis and the profound production of inflammatory cytokines such as TGF-beta, IL-6 and the highly inflammatory IL-17 in melanocytes (Daniels et al, 2004). The ability of eHSP70 to bind to several receptors alongside being in high concentration could explain this effect. However, several studies have found that eHSP70 is able to stimulate anti-inflammatory pathways, alongside being an inhibitor for the NF-kB signalling pathway; therefore, it could be hypothesised that high concentrations of eHSP70 binding to toll-like receptors are upregulating the production of cytokines, resulting in a cytotoxic effect.

4.1.2 Effect of U937 conditioned media on C2C12 cell viability:

The first set of these experiments determined that RPMI itself can induce significant decreases in C2C12 cell viability (figure 3.19). A decrease seen when treating with RPMI could potentially be due to the fact C2C12 murine cells were under stress in the media, as the recommended media from the manufacturer is DMEM (C2C12 | ATCC, 2021). Taking this into consideration, the C2C12 cells have not had the optimum growth environment which will consequently impact the viability overall. Conditioned media (CM) was seen to reduce a similar amount to RPMI, suggestive of the reduction in cell viability being due to cell growth conditions and not the contents of the supernatant used to treat the C2C12 cells. These results dictated that future experiments involving U937 conditioned media be conducted in DMEM. In these future experiments, the cell viability of C2C12 cells was seen to be significantly reduced following treatment of conditioned media collected from LPS-stimulated U937 cells (Figure 3.19). LPS is an endotoxin and is found as a component of the cell wall in gram negative bacteria, it is highly immunogenic and has a profound effect on the activation of immune cells with CD14/TLR4 receptors (Yücel et al., 2017). Vogel et al. found that LPS is potent inducer of inflammatory cytokines such as TNF-a, cyclooxygense-1 and IL-6, therefore the supernatant could cause inflammatory stress upon the C2C12 cells resulting in apoptosis and necrosis ((Vogel et al., 2012; Place and Kanneganti, 2019). In the experiments presented in Figure 3.19, it is interesting that just 1 hour of LPS treatment causes a change in the conditioned medium that can have a profound effect when subsequently applied to C2C12s; the initial response of the U937s to the LPS treatment will be the production of pro-inflammatory cytokines and extracellular HSPs, but it would be interesting to investigate the time period associated with the transcription, translation and trafficking of these events. Indeed a study conducted by Morton et al found that increased Hsp70 expression was undetectable up until 24 hours of stimuli, suggesting a 24 hour period is required to initiate HSP production and release (Morton et al., 2006). The decrease in viability could be indicative of unknown cytotoxic molecules in the supernatant which interestingly reduce cell viability. However, allowing a 24 hour recovery period for the U937 cells, as opposed to the 1 hour, before extracting the supernatant and treating the C2C12 cells displayed increased viability; this could be due to molecules such as eHSP70 initiating a repair and recovery mechanism.

4.2 Changes in myocyte cellular respiration

Non-mitochondrial respiration is representative of oxygen consumption by cellular processes other than the mitochondria, this can be due to detoxification and desaturation enzymes, but is not limited to that. Approximately 10% of non-mitochondrial oxygen consumption is accounted for by

desaturation and detoxification enzymes (Chacko et al., 2014). eHSPs possess the ability to induce inflammatory cytokines such TNF-α, IL-17 and IL-6, this results in the production of ROS (Asea et al., 2000) . ROS are able to deteriorate the outer mitochondrial membrane causing oxygen to leave the mitochondria, consequently more oxygen is available for non-mitochondrial oxygen consumption (Murphy, 2008). Figure 3.4A displays an increase in non-mitochondrial oxygen consumption when treated with 5ng/ml, this could potentially be due to a potential cytotoxic effect of eHSPs at low concentrations. HSPs are constitutively expressed intracellularly, chaperoning proteins (Relja and Land, 2019). However they are released into extracellular space when a stress stimulus is registered (Hightower and Guidon, 1989). One could hypothesise this is since a low concentration of eHSPs bind TLR-4 inducing the production of cytokines, resulting in a high concentration of ROS present in the cell. As there is no stress stimulus present, HSF remains inactive and HSP production is not unregulated in contrast to cytokine production, therefore allowing ROS to have a profound effect on the cell. ROS production causes oxidative damage to mitochondrial proteins, membranes and DNA therefore impairing synthesis of ATP and other metabolic functions. The resulting affect is upregulated expression of detoxification enzymes consuming more oxygen. Figure 3.4B shows a statistically insignificant increase in non-mitochondrial respiration, whereas it remains unchanged with 5, 25 and 50ng/ml. Despite Hsp60 belonging to the group of danger signalling molecules, it is able to induce cellular cytokine synthesis such Interferon-gamma and TNF-alpha; resulting in the production of ROS (Pockley et al., 2008). As mentioned before ROS are able to damage mitochondrial membranes, proteins and DNA; altering the mitochondrial membrane could result in an increased amount of substrate oxygen leaving the mitochondria and consumed by enzymes breaking down organelles and detoxification enzymes stabilising ROS present in the cytosolic space (Capranico et al., 1986). TAK242 was able to decrease NMOC across all treatments, however there was no statistical significance. Complete inhibition of TLR-4 could potentially reduce the level of detoxification enzyme, hence reducing NMOC, however there is very little literature to support this (Zandi et al., 2019).

Basal respiration is largely affected by ATP demand as it is controlled by ATP usage, while a partial role is played by substrate oxidation and proton leak. Therefore, a significant change in maximum respiratory capacity or proton leak will impact basal respiration. Figure 3.5A shows the basal respiration to increase when treated when treated with 5 and 25ng/ml, this could be due to the ability of Hsp70 to induce cytokine, TNF-alpha, which results in the production of NO. NO is known to damage the mitochondrial membrane and cardiolipin, a constituent of mitochondrial membranes in the mitochondria. Similarly, figure 3.5B shows an increase of basal respiration which again could be due the ability of Hsp60 inducing TNF-alpha. A decrease in basal respiration is displayed in figure 3.5a, one

potential mechanism causing this could mitochondrial stress caused by a decrease of calcium ions. Calcium ions communicate with mitochondrial Ca2+ uniporter (MCU) which activates mitochondrial dehydrogenase and ATP synthesis; therefore it could be hypothesised eHSP70 impacts the calcium ion concentration, causing a reduce activity MCU resulting in reduced ATP production and consequently lowering basal respiration (Tarasov et al., 2012). Figure 3.12 shows an increase in basal respiration over control across all treatments, the largest increase seen when treated Hsp60 + TAK242; this could potentially occur due to ROS induced by inflammatory cytokines, the oxidative damage causing increased proton leak therefore increasing basal respiration (Hahn et al., 2014).

Maximal respiration is representative of the maximum capacity of the electron transport chain, this can be visualised using FCCP, despite the increased demand of energy, substrate supply does not increases therefore resulting in a lack of ATP. Maximal respiration increase is indicative of mitochondrial dysfunction. Figure 3.6A shows there to be no significant increase of maximal respiration and this could be due to the protective mechanisms of AMPK. AMP-activated protein kinase is activated when intracellular level of adenosine monophosphate (AMP) is increased due to ATP hydrolysis. AMPK shuts down anabolic pathways and initiates catabolic pathways to produce ATP (Steinberg and Kemp, 2009). Song and his team found that AMPK is required for mitochondrial biogenesis (Zong et al., 2002), a process in which mitochondria grow in their size and number. Figure 3.6B shows an increase of maximal respiration when treated with 10ng/ml. eHsp60 is known to induce the production of cellular cytokines, which in turn activate iNOS causing the production of NO, which is able to damage the mitochondrial membrane and inhibit the respiratory chain, consequently resulting in mitochondrial dysfunction (Poderoso et al., 2019). Furthermore, NO directly impacts cardiolipin, a part of the membrane which the proton passes through back to intramembrane space ensuring an electrochemical gradient is maintained. Damaged and reduced cardiolipin would imminently cause maximal respiration to increase as the electron transport chain cannot function effectively in the absence of an electrochemical gradient (Wilson and Hunt, 2002). When treated with Hsp60 + TAK242, a statistically insignificant increase maximal respiration; taking into consideration TAK242's role as a TLR-4 inhibitor; stimulation of the coreceptor CD40 can stimulate the production of TNF-α (Elgueta et al., 2009), TNF-a causes induces the production of ROS, resulting in a loss of available substrate due to membrane oxidation allowing 'leaking of substrate' (Suematsu et al., 2003).

Protons help to establish an electrochemical gradient on the inner mitochondrial membrane, which enables substrate oxygen to be used on ADP phosphorylation/ATP generation. For this reason, protons are crucial for the continual production of ATP (Mitchel, 1961). A statistically insignificant

increase was seen in both figure 3.7A and 3.7B when treated with 5ng/ml or 10ng/ml. This could be due to the ability of both HSP60 and 70 to induce cellular cytokines which in turn cause the production of ROS, resulting in phospholipid oxidation. As a result of this proton leak is increased, electrical resistance is decreased and the membrane thickness is reduced (Brookes, 2005). Lipid oxidation is a self-propagating reaction therefore can cause severe damage, resulting in an increase proton loss (Ayala et al., 2014). Figure 3.14 shows a significant increase in proton leak when treated with Hsp60 + TAK242, a potential cause for this could be CD14s role as a co-receptor of TLR-4, a study found that unregulated activation of this coreceptor increases inflammation (Fassbender et al., 2003). Increased inflammation caused by cytokines causes oxidative damage to the cardiolipin, causing an increase in proton leak.

ATP is the immediate power source for a cell and is formed by phosphorylating ADP with a phosphate molecule, supported by an electrochemical gradient created by protons moving across the cardiolipin. Healthy cells display an unaffected production of ATP, whereas cells experiencing mitochondrial dysfunction will have reduced ATP production. Figure 3.8A shows an insignificant decrease in ATP production when treated with 10 and 50ng/ml, this could be caused by Hsp70 inducing IL-17 (Kottke et al., 2007) which in study conducted by Kim et al displayed inhibitory functions on the expression of Oxidative phosphorylation systems (OxPhos) components, causing a reduction in ATP production (Kim et al., 2017). Figure 3.8B shows a statistically insignificant increase of ATP production when treated with 10ng/ml, one possible mechanism for this was found by Marino Gammazza et al (Marino Gammazza et al., 2018), Hsp60 is able to induce the phosphorylation of AMPK which activates the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1a) which is known to be the ‘master regulator’ of mitochondrial biogenesis (White et al., 2013). Increase of mitochondrial biogenesis would consequently result in greater production of mitochondrion therefore increasing ATP production. Figure 3.15 displays a statistically insignificant increase ATP production, it can be hypothesised the inhibition of TLR and increased binding to other receptors such as CD14, CD40 is inducing changes to the energy demand of the cell (Yi and Bishop, 2014)

SRC is the ability of the substrate supply and electron transport to respond to changes in energy requirement and a reduced SRC is indicative of mitochondrial dysfunction. SRC is mediated by the integrity of the respiratory chain and proton permeability. Although different cell types would have different effects, the respiratory chain components play a crucial role in SRC maintenance (Carbognin et al., 2016). It was found that complex I and complex II both maintain SRC levels however SRC levels still increased when complex I was inhibited. It was found in myeloid leukaemia cells that reduced

enzymatic activity of complex III reduced SRC levels (Sriskanthadevan et al., 2012). Another crucial factor controlling SRC is proton permeability. The presence of the phospholipid cardiolipin is essential in ensuring a proton gradient is is maintained which is paramount for ATP synthesis. Taking this into consideration, regulating inner mitochondrial membrane integrity is key (Nguyen et al., 2016). Figures 3.9A and 3.9B both show SRC to remain similar across all treatments. This could be due to the protective mechanisms of the mitochondrion initiating AMPK signalling pathways in response to ROS production caused by cytokines production through TLR-2/4 signalling. Figure 3.16 displays a small increase when treated with Hsp60, hsp70, Hsp70 + TAK242, however a much larger statistically insignificant increase when treated with hsp60 + TAK242; as TAK242 is an inhibitor of TLR, Hsp60 is more inclined to bind to CD14 and CD40 receptors (Pockley et al., 2008). Binding to these receptors induces defensive mechanisms such as the activation mitogen activated protein kinase and Signal transducers and activators of transcription-3 which could be mechanism responsible for the increase in SRC, however there is no substantial evidence to support this (Yuan et al., 2013).

Coupling Efficacy is the quantification of how effectively of two substates bind together, such as substrate oxygen and ATP. The mitochondria respiratory chain is highly sensitive to oxidative damage so production of ROS will directly impact the coupling efficacy as damaged respiratory chain and mitochondrial membrane will result in the following: increased proton leak into intracellular matrix while movement of electron down the respiratory chain is reduced due to oxidative damage. Consequently, the binding of substrate oxygen and ATP is reduced impacted (Sas et al., 2007). Figure 3.10A shows a small statistically insignificant decrease, this could be due to the cytotoxic effects of TNF-alpha through ROS production. However, coupling efficacy is seen to increase as the dosage of Hsp70 increases, the reasons behind this are unknown but a speculation is that Hsp70 maybe indirectly inducing AMPK activation, which activates PGC-1alpha. Figure 3.10B displays a decrease in coupling efficacy with no statistical significance, thus could be occurring due to the oxidative damaged caused to the mitochondrial membrane resulting in the aforementioned, increased proton leak, resulting in decreased coupling efficacy (Jastroch et al., 2010). Figure 3.17 shows the addition of TAK242 with treatments of Hsp60 and 70, this could be due to TAK242 inhibiting TLR 2 and 4 preventing the production of cellular cytokine production. Consequently, the mitochondrion is not subject to membrane oxidation which maintains the electrochemical gradient allowing the binding of substrate oxygen and ATP (Paradies, 2014).

Xfp mitochondrial stress was also conducted using supernatant acquired by treating U937 cells for 1, 2, 4 and 24 hours. The data shows a statistically significant increase in NMOC, which could babe caused

by the release of COX-1 and lipoxygenase-activating protein from the U937 cells (Kargman et al., 1993), furthermore the NMOC could be increased due to the eHPSs in the supernatant inducing the up regulation of HSP production, this will result in more enzymatic activity such as RNA polymerase. As a result of increased NMOC, the energy demand will also increase. This is reflected in figures 3.21 and 3.24, where the basal respiration and ATP production have increased significantly to see the requirements of the cellular energy demand. Cellular energy demand can increase when due to the increase of enzymatic activity, we have already discovered that LPS is able to induce the production of COX-1 and 5-lipoxygenase activating protein (FLAP) (Vogel et al., 2012)(Kargman et al., 1993), furthermore, it was speculated that supernatant would be HSP-rich; considering this is the case then it can be hypothesised there is an increase of protein synthesis due to increased HSP stimulation. Figure 3.25 is indicative of SRC depleting, which could be due to the increased cellular energy demand as the rate of ATP Production and NMOC is increased.

4.3 Limitations and improvements:

The following limitations presented in the study. The first limitation of this study was the inability to conduct protein assay on conditioned media to identify whether HSPs were present in the media and which cytokines were released during LPS treatments, cell viability was reduced during the first set of experiments with U937 cells. When given the manufacture recommended media and 24-hour recovery period, cell viability was seen to increase. In depth protein analysis would allow us to highlight any molecules which could potentially have a cytotoxic effect, decreasing the cell viability. Future experiments should entail using DMEM (BE12-604) when culturing cells, as U937 cells are very robust and are able to grow in various media (Chanput et al., 2015), whereas C2C12 cells are sensitive to the growth media in which they are inoculated. Another limitation was the large variance of 10ng/ml of HSP70 results, this was due to experimental error and repeating the experiment was required, however due to time constraints this was not possible. Another limitation of this study was assessing the effect of TAK242 on cell viability, as this would highlight whether the inhibitory effect of TAK242 was able to proliferate C2C12 cell viability. The third limitation presented in the form of HSPs having no effect on cellular respiration, this being a limitation as it is hindering the effect of TLR-inhibitor TAK242 and cannot illustrate the inhibitory effects. To improve the quality of data derived from this study, protein assays should be carried out to ensure the presence of HSP release into extracellular environment, identify cytotoxic proteins which could also be contributing to the decreased cell viability and what type of cytokines are present. Understanding this will enable us to understand the mechanism behind reduction of cell viability as HSPs effects are dependent on the origin of cell releasing the protein and the receptor it will bind to. Another limitation of this study is the fact the cells do not effectively represent a multinucleated environment, like that of a muscle

environment. This can be overcome by maturing the myocytes into myoblasts, which would be more representative of a muscle environment.

4.4 Future implications:

When taking into account the incidence rate of ICUAW and its lack of effective treatments, there is need for investigation for more effective treatment. The findings of studies may not directly present the treatment to help eliminate ICUAW; but surely does cast light on a potential mechanism taking place. Further studying the effects of heat shock proteins on a multinucleated structure would potential bring to light the exact mechanism to be targeted for therapeutic interventions. Additionally, a combination of protein assays and cellular function analysis would provide significant data for the prevention of ICUAW, as it could bring to light potential signalling molecules leading up to the onset of ICUAW; allowing early prevention.

5.0 Conclusion

In conclusion, recombinant Hsp70, when applied exogenously to C2C12 murine myocytes were found to cause a significant reduction in cell viability, and this effect was shown to be dose dependent. Recombinant Hsp60 when applied exogenously also appeared to have some effect on cell viability, particularly at a concentration of 5ng/ml, but certainly not to the same degree as Hsp70.

Despite their apparent cytotoxic effects, neither recombinant Hsp70 or Hsp60 when applied exogenously had any significant effects on any aspects of cellular respiration, including non-mitochondrial respiration, basal respiration, maximal respiration, proton leak, ATP production, spare respiratory capacity or coupling efficiency.

When an inhibitor of TLR-4, TAK242, was applied to the cells in combination with the HSPs, there was also no effect on any aspects of cellular respiration.

Conditioned media from a monocyte-derived cell line, U937, that had been pre-stimulated with LPS was found to have significant effects on both the C2C12 cell viability and on aspects of C2C12 cellular respiration, including a significant increase in non-mitochondrial respiration, basal respiration and ATP production, and a significant reduction in spare respiratory capacity.

Further work is needed to determine the inflammatory profile of the U937 secretome following LPS simulation, to determine the precise factors that may be responsible for this change in metabolism.

However, since the use of recombinant Hsp70 and Hsp60 did not show any effects on aspects of metabolism, further work will be required to understand if there is any link between the high levels of extracellular HSPs found in critically ill patients, and the development of muscle ICU-acquired muscle weakness. Certainly, the work presented here would point to other factors as a stronger cause.

Bibliography:

Asea, A., Kraeft, S., Kurt-Jones, E., Stevenson, M., Chen, L., Finberg, R., Koo, G. and Calderwood, S. (2000) 'HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine'. *Nature Medicine*, 6(4) pp.435-442.

Batulan, Z., Pulakazhi Venu, V., Li, Y., Koumbadinga, G., Alvarez-Olmedo, D., Shi, C. and O'Brien, E. (2016) 'Extracellular Release and Signaling by Heat Shock Protein 27: Role in Modifying Vascular Inflammation'. *Frontiers in Immunology*, 7.

Bausero, M., Gastpar, R., Multhoff, G. and Asea, A. (2005) 'Alternative Mechanism by which IFN-γ Enhances Tumor Recognition: Active Release of Heat Shock Protein 72'. *The Journal of Immunology*, 175(5) pp.2900-2912.

Biolo, G., Tipton, K., Klein, S. and Wolfe, R. (1997) 'An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein'. *American Journal of Physiology-Endocrinology and Metabolism*, 273(1) pp.E122-E129.

Bolton, C. (2005) 'Neuromuscular manifestations of critical illness'. *Muscle & Nerve*, 32(2) pp.140-163.

Brookes, P. (2005) 'Mitochondrial H+ leak and ROS generation: An odd couple'. *Free Radical Biology and Medicine*, 38(1) pp.12-23.

Capranico, G., Dasdia, T. and Zunino, F. (1986) 'Comparison of doxorubicin-induced DNA damage in doxorubicin-sensitive and -resistant P388 murine leukemia cells'. *International Journal of Cancer*, 37(2) pp.227-231.

Carbognin, E., Betto, R., Soriano, M., Smith, A. and Martello, G. (2016) 'Stat3 promotes mitochondrial transcription and oxidative respiration during maintenance and induction of naive pluripotency'. *The EMBO Journal*, 35(6) pp.618-634.

Cell Counting Kit 8 (WST-8 / CCK8) (ab228554) | Abcam. (2021) Abcam.com. [Online] [Accessed on 14 December 2021] https://www.abcam.com/cell-counting-kit-8-wst-8--cck8-ab228554.html.

Chacko, B., Kramer, P., Ravi, S., Benavides, G., Mitchell, T., Dranka, B., Ferrick, D., Singal, A., Ballinger, S., Bailey, S., Hardy, R., Zhang, J., Zhi, D. and Darley-Usmar, V. (2014) 'The Bioenergetic Health Index: a new concept in mitochondrial translational research'. *Clinical Science*, 127(6) pp.367-373.

Chanput, W., Peters, V. and Wichers, H. (2015) 'THP-1 and U937 Cells'. *The Impact of Food Bioactives on Health*, pp.147-159.

Colaco, C., Bailey, C., Walker, K. and Keeble, J. (2013) 'Heat Shock Proteins: Stimulators of Innate and Acquired Immunity'. *BioMed Research International*, 2013 pp.1-11.

Chen, S., Bawa, D., Besshoh, S., Gurd, J. and Brown, I. (2005) 'Association of heat shock proteins and neuronal membrane components with lipid rafts from the rat brain'. *Journal of Neuroscience Research*, 81(4) pp.522-529.

Daniels, G., Sanchez-Perez, L., Diaz, R., Kottke, T., Thompson, J., Lai, M., Gough, M., Karim, M., Bushell, A., Chong, H., Melcher, A., Harrington, K. and Vile, R. (2004) 'A simple method to cure established tumors by inflammatory killing of normal cells'. *Nature Biotechnology*, 22(9) pp.1125-1132.

Dave, H., Shook, M. and Varacallo, M., 2021. *Anatomy, Skeletal Muscle*. [online] Ncbi.nlm.nih.gov. Available at: <https://www.ncbi.nlm.nih.gov/books/NBK537236/> [Accessed 18 November 2021].

Dirks, M., Hansen, D., Van Assche, A., Dendale, P. and Van Loon, L. (2014) 'Neuromuscular electrical stimulation prevents muscle wasting in critically ill comatose patients'. *Clinical Science*, 128(6) pp.357-365.

Elgueta, R., Benson, M., de Vries, V., Wasiuk, A., Guo, Y. and Noelle, R. (2009) 'Molecular mechanism and function of CD40/CD40L engagement in the immune system'. *Immunological Reviews*, 229(1) pp.152-172.

Fassbender, K., Walter, S., Kühl, S., Landmann, R., Ishii, K., Bertsch, T., Stalder, A., Muehlhauser, F., Liu, Y., Ulmer, A., Rivest, S., Lentschat, A., Gulbins, E., Jucker, M., Staufenbiel, M., Brechtel, K., Walter, J., Multhaup, G., Penke, B., Adachi, Y.,

Fanzani, A., Conraads, V., Penna, F. and Martinet, W. (2012) 'Molecular and cellular mechanisms of skeletal muscle atrophy: an update'. *Journal of Cachexia, Sarcopenia and Muscle*, 3(3) pp.163-179.

Hartmann, T. and Beyreuther, K. (2003) 'The LPS receptor (CD14) links innate immunity with Alzheimer's disease'. *The FASEB Journal*, 18(1) pp.203-205.

Hayden, M. and Ghosh, S. (2008) 'Shared Principles in NF-κB Signaling'. *Cell*, 132(3) pp.344-362.

Herridge, M. (2002) 'Long-term outcomes after critical illness'. *Current Opinion in Critical Care*, 8(4) pp.331-336.

Hightower, L. and Guidon, P. (1989) 'Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins'. *Journal of Cellular Physiology*, 138(2) pp.257-266.

Hill, B., Benavides, G., Lancaster, J., Ballinger, S., Dell'Italia, L., Zhang, J. and Darley-Usmar, V. (2012) 'Integration of cellular bioenergetics with mitochondrial quality control and autophagy'. *Biological Chemistry*, 393(12) pp.1485-1512.

HWANG, S., KANG, Y., SUNG, B., KIM, M., KIM, D., LEE, Y., YOO, M., KIM, C., CHUNG, H. and KIM, N. (2015) 'Folic acid promotes the myogenic differentiation of C2C12 murine myoblasts through the Akt signaling pathway'. *International Journal of Molecular Medicine*, 36(4) pp.1073-1080.

Fajgenbaum, D. and June, C. (2020) 'Cytokine Storm'. *New England Journal of Medicine*, 383(23) pp.2255-2273.

Friedrich, O., Reid, M., Van den Berghe, G., Vanhorebeek, I., Hermans, G., Rich, M. and Larsson, L. (2015) 'The Sick and the Weak: Neuropathies/Myopathies in the Critically Ill'. *Physiological Reviews*, 95(3) pp.1025-1109.

Gastpar, R., Gehrmann, M., Bausero, M., Asea, A., Gross, C., Schroeder, J. and Multhoff, G. (2005) 'Heat Shock Protein 70 Surface-Positive Tumor Exosomes Stimulate Migratory and Cytolytic Activity of Natural Killer Cells'. *Cancer Research*, 65(12) pp.5238-5247.

Giraldo, E., Martin-Cordero, L., Garcia, J., Gerhmann, M., Multhoff, G. and Ortega, E. (2009) 'Exercise-induced extracellular 72 kDa heat shock protein (Hsp72) stimulates neutrophil phagocytic and fungicidal capacities via TLR-2'. *European Journal of Applied Physiology*, 108(2) pp.217-225.

Glass, D. (2003) 'Molecular mechanisms modulating muscle mass'. *Trends in Molecular Medicine*, 9(8) pp.344-350.

Jackman, R., Cornwell, E., Wu, C. and Kandarian, S. (2012) 'Nuclear factor-κB signalling and transcriptional regulation in skeletal muscle atrophy'. *Experimental Physiology*, 98(1) pp.19-24.

Jastroch, M., Divakaruni, A., Mookerjee, S., Treberg, J. and Brand, M. (2010) 'Mitochondrial proton and electron leaks'. *Essays in Biochemistry*, 47 pp.53-67.

Jolly, C. (2000) 'Role of the Heat Shock Response and Molecular Chaperones in Oncogenesis and Cell Death'. *Journal of the National Cancer Institute*, 92(19) pp.1564-1572.

Kandel, J., Angelin, A., Wallace, D. and Eckmann, D. (2016) 'Mitochondrial respiration is sensitive to cytoarchitectural breakdown'. *Integrative Biology*, 8(11) pp.1170-1182.

Kargman, S., Rousseau, P., Reid, G., Rouzer, C., Mancini, J., Rands, E., Dixon, R., Diehl, R., Leville, C. and Nathaniel, D. (1993) 'Leukotriene synthesis in U937 cells expressing recombinant 5-lipoxygenase'. *J Lipid Mediat*, 7(1) pp.31-24.

Klumpen, E., Hoffschröer, N., Zeis, B., Gigengack, U., Dohmen, E. and Paul, R. (2016) 'Reactive oxygen species (ROS) and the heat stress response ofDaphnia pulex: ROS-mediated activation of hypoxia-inducible factor 1 (HIF-1) and heat shock factor 1 (HSF-1) and the clustered expression of stress genes'. *Biology of the Cell*, 109(1) pp.39-64.

Kohlmeier, M. (2015) *Nutrient Metabolism*. Elsevier Science.

Kottke, T., Sanchez-Perez, L., Diaz, R., Thompson, J., Chong, H., Harrington, K., Calderwood, S., Pulido, J., Georgopoulos, N., Selby, P., Melcher, A. and Vile, R. (2007) 'Induction of hsp70-Mediated Th17 Autoimmunity Can Be Exploited as Immunotherapy for Metastatic Prostate Cancer'. *Cancer Research*, 67(24) pp.11970-11979.

Kraner, S., Novak, K., Wang, Q., Peng, J. and Rich, M. (2012) 'Altered sodium channel-protein associations in critical illness myopathy'. *Skeletal Muscle*, 2(1).

Krasnoff, J. and Painter, P., 1999. The Physiological Consequences of Bed Rest and Inactivity. *Advances in Renal Replacement Therapy*, 6(2), pp.124-132.

Kriegenburg, F., Ellgaard, L. and Hartmann-Petersen, R. (2012) 'Molecular chaperones in targeting misfolded proteins for ubiquitin-dependent degradation'. *FEBS Journal*, 279(4) pp.532-542.

Kurutas, E. (2015) 'The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state'. *Nutrition Journal*, 15(1).

Latronico, N., Recupero, D., Candiani, A., Guarneri, B., De Maria, G., Antonini, L., Fenzi, F., Tomelleri, G., Tonin, P. and Rizzuto, N. (1996) 'Critical illness myopathy and neuropathy'. *The Lancet*, 347(9015) pp.1579-1582.

Lecker, S. (2003) 'Ubiquitin-protein ligases in muscle wasting: multiple parallel pathways?'. *Current Opinion in Clinical Nutrition & Metabolic Care*, 6(3) pp.271-275.

Lee, D., Santomasso, B., Locke, F., Ghobadi, A., Turtle, C., Brudno, J., Maus, M., Park, J., Mead, E., Pavletic, S., Go, W., Eldjerou, L., Gardner, R., Frey, N., Curran, K., Peggs, K., Pasquini, M., DiPersio, J., van den Brink, M., Komanduri, K., Grupp, S. and Neelapu, S. (2019) 'ASTCT Consensus Grading for Cytokine Release Syndrome and Neurologic Toxicity Associated with Immune Effector Cells'. *Biology of Blood and Marrow Transplantation*, 25(4) pp.625-638.

Levine, S., Biswas, C., Dierov, J., Barsotti, R., Shrager, J., Nguyen, T., Sonnad, S., Kucharchzuk, J., Kaiser, L., Singhal, S. and Budak, M. (2011) 'Increased Proteolysis, Myosin Depletion, and Atrophic AKT-FOXO Signaling in Human Diaphragm Disuse'. *American Journal of Respiratory and Critical Care Medicine*, 183(4) pp.483-490.

Li, Y., Chen, Y., John, J., Moylan, J., Jin, B., Mann, D. and Reid, M. (2005) 'TNF-α acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle'. *The FASEB Journal*, 19(3) pp.362-370.

Li, Y., Schwartz, R., Waddell, I., Holloway, B. and Reid, M. (1998) 'Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-κB activation in response to tumor necrosis factorα'. *The FASEB Journal*, 12(10) pp.871-880.

Luo, X., Zuo, X., Zhou, Y., Zhang, B., Shi, Y., Liu, M., Wang, K., McMillian, D. and Xiao, X. (2008) 'Extracellular heat shock protein 70 inhibits tumour necrosis factor-α induced proinflammatory mediator production in fibroblast-like synoviocytes'. *Arthritis Research & Therapy*, 10(2) p.R41.

Madamanchi, N., Li, S., Patterson, C. and Runge, M. (2001) 'Reactive Oxygen Species Regulate Heat-Shock Protein 70 via the JAK/STAT Pathway'. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 21(3) pp.321-326.

Mambula, S. and Calderwood, S. (2006) 'Heat Shock Protein 70 Is Secreted from Tumor Cells by a Nonclassical Pathway Involving Lysosomal Endosomes'. *The Journal of Immunology*, 177(11) pp.7849-7857.

Marino Gammazza, A., Macaluso, F., Di Felice, V., Cappello, F. and Barone, R. (2018) 'Hsp60 in Skeletal Muscle Fiber Biogenesis and Homeostasis: From Physical Exercise to Skeletal Muscle Pathology'. *Cells*, 7(12) p.224.

McArdle, A., H. Dillmann, W., Mestril, R., A. Faulkner, J. and J. Jackson, M. (2003) 'Overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction'. *The FASEB Journal*, 18(2) pp.1-12.

McCord, J. (1988) 'Free radicals and myocardial ischemia: Overview and outlook'. *Free Radical Biology and Medicine*, 4(1) pp.9-14.

Meesen, R., Dendale, P., Cuypers, K., Berger, J., Hermans, A., Thijs, H. and Levin, O. (2010) 'Neuromuscular Electrical Stimulation As a Possible Means to Prevent Muscle Tissue Wasting in Artificially Ventilated and Sedated Patients in the Intensive Care Unit: A Pilot Study'. *Neuromodulation: Technology at the Neural Interface*, 13(4) pp.315-321.

Miyabara, E., Nascimento, T., Rodrigues, D., Moriscot, A., Davila, W., AitMou, Y., deTombe, P. and Mestril, R. (2012) 'Overexpression of inducible 70-kDa heat shock protein in mouse improves structural and functional recovery of skeletal muscles from atrophy'. *Pflügers Archiv - European Journal of Physiology*, 463(5) pp.733-741.

Mizushima, N., Levine, B., Cuervo, A. and Klionsky, D. (2008) 'Autophagy fights disease through cellular self-digestion'. *Nature*, 451(7182) pp.1069-1075.

Morimoto, R. (1998) 'Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators'. *Genes & Development*, 12(24) pp.3788-3796.

Morton, J., Kayani, A., McArdle, A. and Drust, B. (2009) 'The Exercise-Induced Stress Response of Skeletal Muscle, with Specific Emphasis on Humans'. *Sports Medicine*, 39(8) pp.643-662.

Morton, J., MacLaren, D., Cable, N., Bongers, T., Griffiths, R., Campbell, I., Evans, L., Kayani, A., McArdle, A. and Drust, B. (2006) 'Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise'. *Journal of Applied Physiology*, 101(1) pp.176-182.

Murphy, M. (2008) 'How mitochondria produce reactive oxygen species'. *Biochemical Journal*, 417(1) pp.1-13.

Negulyaev, Y., Vedernikova, E., Kinev, A. and Voronin, A. (1996) 'Exogenous heat shock protein hsp70 activates potassium channels in U937 cells'. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1282(1) pp.156-162.

Nguyen, H., Mejia, E., Chang, W., Wang, Y., Watson, E., On, N., Miller, D. and Hatch, G. (2016) 'Reduction in cardiolipin decreases mitochondrial spare respiratory capacity and increases glucose transport into and across human brain cerebral microvascular endothelial cells'. *Journal of Neurochemistry*, 139(1) pp.68-80.

Place, D. and Kanneganti, T. (2019) 'Cell death–mediated cytokine release and its therapeutic implications'. *Journal of Experimental Medicine*, 216(7) pp.1474-1486.

Ochala, J., Gustafson, A., Diez, M., Renaud, G., Li, M., Aare, S., Qaisar, R., Banduseela, V., Hedström, Y., Tang, X., Dworkin, B., Ford, G., Nair, K., Perera, S., Gautel, M. and Larsson, L. (2011) 'Preferential skeletal muscle myosin loss in response to mechanical silencing in a novel rat intensive care unit model: underlying mechanisms'. *The Journal of Physiology*, 589(8) pp.2007-2026.

Paradies, G. (2014) 'Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease'. *World Journal of Gastroenterology*, 20(39) p.14205.

Petersson, B., Wernerman, J., Waller, S., von der Decken, A. and Vinnars, E. (1990) 'Elective abdominal surgery depresses muscle protein synthesis and increases subjective fatigue: Effects lasting more than 30 days'. *British Journal of Surgery*, 77(7) pp.796-800.

Petrov, A., Kravtsova, V., Matchkov, V., Vasiliev, A., Zefirov, A., Chibalin, A., Heiny, J. and Krivoi, I. (2017) 'Membrane lipid rafts are disturbed in the response of rat skeletal muscle to short-term disuse'. *American Journal of Physiology-Cell Physiology*, 312(5) pp.C627-C637.

Pockley, A., Muthana, M. and Calderwood, S. (2008) 'The dual immunoregulatory roles of stress proteins'. *Trends in Biochemical Sciences*, 33(2) pp.71-79.

Profumo, E., Buttari, B., Tinaburri, L., D'Arcangelo, D., Sorice, M., Capozzi, A., Garofalo, T., Facchiano, A., Businaro, R., Kumar, P., Singh, B., Parmar, V., Saso, L. and Riganò, R. (2018) 'Oxidative Stress Induces HSP90 Upregulation on the Surface of Primary Human Endothelial Cells: Role of the Antioxidant 7,8-Dihydroxy-4-methylcoumarin in Preventing HSP90 Exposure to the Immune System'. *Oxidative Medicine and Cellular Longevity*, 2018 pp.1-9.

Poderoso, J., Helfenberger, K. and Poderoso, C. (2019) 'The effect of nitric oxide on mitochondrial respiration'. *Nitric Oxide*, 88 pp.61-72.

Ralph, P., Moore, M. and Nilsson, K. (1976) 'Lysozyme synthesis by established human and murine histiocytic lymphoma cell lines.'. *Journal of Experimental Medicine*, 143(6) pp.1528-1533.

Ritossa, F. (1962) 'A new puffing pattern induced by temperature shock and DNP in drosophila'. *Experientia*, 18(12) pp.571-573.

Rothman, J. and Schekman, R. (2011) 'Molecular Mechanism of Protein Folding in the Cell'. *Cell*, 146(6) pp.851-854.

Tytell, M. and Hooper, P. (2001) 'Heat shock proteins: new keys to the development of cytoprotective therapies'. *Emerging Therapeutic Targets*, 5(2) pp.267-287.

Senf, S. (2013) 'Skeletal muscle heat shock protein 70: diverse functions and therapeutic potential for wasting disorders'. *Frontiers in Physiology*, 4.

Senf, S., Howard, T., Ahn, B., Ferreira, L. and Judge, A. (2013) 'Loss of the Inducible Hsp70 Delays the Inflammatory Response to Skeletal Muscle Injury and Severely Impairs Muscle Regeneration'. *PLoS ONE*, 8(4) p.e62687.

Smolková, K. and Ježek, P. (2012) 'The Role of Mitochondrial NADPH-Dependent Isocitrate Dehydrogenase in Cancer Cells'. *International Journal of Cell Biology*, 2012 pp.1-12.

Sriskanthadevan, S., Chung, T., Skrtic, M., Jhas, B., Hurren, R., Gronda, M., Wang, X., Jitkova, Y., Sukhai, M., Lin, F., Maclean, N., Laister, R., Mullen, P., Xie, S., Penn, L., Rogers, I., Dick, J., Minden, M. and Schimmer, A. (2012) 'AML Cells Have Altered Mitochondrial Biogenesis and Low Spare Reserve Capacity in Their Respiratory Chain That Renders Them Susceptible to Oxidative Metabolic Stress.'. *Blood*, 120(21) pp.2581-2581.

Steinberg, G. and Kemp, B. (2009) 'AMPK in Health and Disease'. *Physiological Reviews*, 89(3) pp.1025-1078.

Stitt, T., Drujan, D., Clarke, B., Panaro, F., Timofeyva, Y., Kline, W., Gonzalez, M., Yancopoulos, G. and Glass, D. (2004) 'The IGF-1/PI3K/Akt Pathway Prevents Expression of Muscle Atrophy-Induced Ubiquitin Ligases by Inhibiting FOXO Transcription Factors'. *Molecular Cell*, 14(3) pp.395-403.

Sorger, P. (1991) 'Heat shock factor and the heat shock response'. *Cell*, 65(3) pp.363-366.

Suematsu, N., Tsutsui, H., Wen, J., Kang, D., Ikeuchi, M., Ide, T., Hayashidani, S., Shiomi, T., Kubota, T., Hamasaki, N. and Takeshita, A. (2003) 'Oxidative Stress Mediates Tumor Necrosis Factor-α–Induced Mitochondrial DNA Damage and Dysfunction in Cardiac Myocytes'. *Circulation*, 107(10) pp.1418-1423.

Tarasov, A., Griffiths, E. and Rutter, G. (2012) 'Regulation of ATP production by mitochondrial Ca2+'. *Cell Calcium*, 52(1) pp.28-35.

van Hees, H., Schellekens, W., Linkels, M., Leenders, F., Zoll, J., Donders, R., Dekhuijzen, P., van der Hoeven, J. and Heunks, L. (2011) 'Plasma from septic shock patients induces loss of muscle protein'. *Critical Care*, 15(5) p.R233.

Vasilaki, A., Jackson, M. and McArdle, A. (2002) 'Attenuated HSP70 response in skeletal muscle of aged rats following contractile activity'. *Muscle & Nerve*, 25(6) pp.902-905.

Venditti, P., Napolitano, G. and Di Meo, S. (2015) 'Role of Mitochondria and Other ROS Sources in Hyperthyroidism-Linked Oxidative Stress'. *Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry*, 15(1) pp.5-36.

Vogel, C., Garcia, J., Wu, D., Mitchell, D., Zhang, Y., Kado, N., Wong, P., Trujillo, D., Lollies, A., Bennet, D., Schenker, M. and Mitloehner, F. (2012) 'Activation of inflammatory responses in human U937 macrophages by particulate matter collected from dairy farms: an in vitro expression analysis of pro-inflammatory markers'. *Environmental Health*, 11(1).

White, J., Ruas, J., Rao, R., Kleiner, S., Wu, J. and Spiegelman, B. (2013) 'A novel PGC-1á isoform induced by resistance training regulates skeletal muscle hypertrophy'. *The FASEB Journal*, 27(S1).

Wilson, J. and Hunt, T. (2002) *Molecular biology of the cell, 4th edition*. New York: Garland Science.

Wei, Y., Zhao, X., Kariya, Y., Teshigawara, K. and Uchida, A. (1995) 'Inhibition of proliferation and induction of apoptosis by abrogation of heat-shock protein (HSP) 70 expression in tumor cells'. *Cancer Immunology Immunotherapy*, 40(2) pp.73-78.

Wolfe, R. (2005) 'Regulation of skeletal muscle protein metabolism in catabolic states'. *Current Opinion in Clinical Nutrition and Metabolic Care*, 8(1) pp.61-65.

Wu, C. (1995) 'Heat Shock Transcription Factors: Structure and Regulation'. *Annual Review of Cell and Developmental Biology*, 11(1) pp.441-469.

Yi, Z. and Bishop, G. (2014) 'Regulatory role of CD40 in obesity-induced insulin resistance'. *Adipocyte*, 4(1) pp.65-69.

Yuan, L., Wang, J., Xiao, H., Wu, W., Wang, Y. and Liu, X. (2013) 'MAPK signaling pathways regulate mitochondrial-mediated apoptosis induced by isoorientin in human hepatoblastoma cancer cells'. *Food and Chemical Toxicology*, 53 pp.62-68.

Yücel, G., Zhao, Z., El-Battrawy, I., Lan, H., Lang, S., Li, X., Buljubasic, F., Zimmermann, W., Cyganek, L., Utikal, J., Ravens, U., Wieland, T., Borggrefe, M., Zhou, X. and Akin, I. (2017) 'Lipopolysaccharides induced inflammatory responses and electrophysiological dysfunctions in human-induced pluripotent stem cell derived cardiomyocytes'. *Scientific Reports*, 7(1).

Zandi, Z., Kashani, B., Poursani, E., Bashash, D., Kabuli, M., Momeny, M., Mousavi-pak, S., Sheikhsaran, F., Alimoghaddam, K., Mousavi, S. and Ghaffari, S. (2019) 'TLR4 blockade using TAK-242 suppresses ovarian and breast cancer cells invasion through the inhibition of extracellular matrix degradation and epithelial-mesenchymal transition'. *European Journal of Pharmacology*, 853 pp.256-263.

Zong, H., Ren, J., Young, L., Pypaert, M., Mu, J., Birnbaum, M. and Shulman, G. (2002) 'AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation'. *Proceedings of the National Academy of Sciences*, 99(25) pp.15983-15987.

Åkerfelt, M., Morimoto, R. and Sistonen, L. (2010) 'Heat shock factors: integrators of cell stress, development and lifespan'. *Nature Reviews Molecular Cell Biology*, 11(8) pp.545-555.) pp.545-555.