Capsaicin protects against atrophy in human skeletal muscle cells

MPhil Thesis by

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Abstract

Skeletal muscle atrophy occurs in many pathological conditions, e.g. AIDS, cancer, sepsis and starvation, and with increased age. There is currently no effective treatment to prevent or reverse this muscle wasting. The TRPV1 agonist, capsaicin, has previously been shown to have a protective effect against skeletal muscle atrophy in mice as well as stimulate hypertrophy. We therefore investigated the effects of capsaicin against dexamethasone-induced atrophy in human primary skeletal muscle myotubes. By treating myotubes with 50μM dexamethasone we successfully induced atrophy, and saw a significant decrease in total protein content as well as MYH2 expression without a change in the atrophy genes BNIP3, GABARAPL1 and FBXO32. 100nM capsaicin treatment in isolation had no effect on protein content but significantly elevated the expression of MYH2 and MYOG above that of dexamethasone-treated cells as well as untreated control. However when combined with dexamethasone, capsaicin reduced some of the negative effects seen previously with dexamethasone alone. The addition of TNFα to the cell culture medium failed to induce atrophy in these myotubes. From the findings of this initial experiment it can be conclude that capsaicin has the capacity to protect against dexamethasone-induced atrophy in these human skeletal myotubes.

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Authors Declaration

I hereby declare this thesis to be my own work except for where acknowledgement has been given.

Signature:

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List of Abbreviations

AIDS- acquired immune deficiency syndrome

BNIP3- BCL2/adenovirus E1B 19kDa protein-interacting protein 3

$[Ca^{2+}]_i$ - intracellular calcium levels

COPD- Chronic Obstructive Pulmonary Disease

DM- differentiation medium

DUBs- deubiquiting enzymes

EDL- extensor digitorum longus

eIF3-f -eukaryotic translation initiation factor 3 subunit-F

eIF4A- eukaryotic translation initiation factor 4A

eIF4E- eukaryotic translation initiation factor 4E

eIF4F- the eukaryotic translation initiation factor 4F

eIF4G- eukaryotic translation initiation factor 4G

E1- ubiquitin-activating enzymes

E2- ubiquitin-conjugating enzyme

E3- ubiquitin protein ligase

4E-BP1- eukaryotic translation initiation factor 4E binding protein 1

FBXO32/MAFbx1/Atrogin-1- F-Box Protein 32

FBXO40- F-box protein 40

FOXOs- Forkhead Box, subunit O transcription factors

GABARAPL1- gamma-aminobutyric acid receptor-associated protein-like 1

GM- growth medium

HPRT1- hypoxanthine phosphoribosyltransferase 1

Hsc70- Heat shock cognate 70

IGF-1- Insulin-like growth factor 1

IRS1- Insulin receptor substrate 1.

KLF15- Kruppel-like factor-15

Lamp2a- lysosomal-associated membrane protein 2

LC3- microtubule-associated protein 1 light chain 3

MRFs- myogenic regulatory factors

MRF4- myogenic regulatory factor 4

mTOR- mammalian target of rapamycin

mTORC1- mTOR complex 1

mTORC2- mTOR complex 2

Mul1- mitochondrial E3 ubiquitin protein ligase 1

MuRF1-Muscle RING-finger protein 1

MYH2- myosin heavy chain-2

Myf5- myogenic factor 5

MYOG- myogenin

MYOD/MyoD- myoblast determination protein 1

NF-$_K$B- nuclear factor of kappa light polypeptide gene enhancer in B-cells

nNOS- neuronal nitric oxide synthase

PGC-1α- peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

PIP3- plasma membrane intrinsic protein 3

PI3K- phosphatidylinositol-4, 5-bisphosphate 3-kinase

p38 MAPK- p38 mitogen-activated protein kinase

p70S6K- ribosomal protein S6 kinase, 70kDa, polypeptide 1

REDD1- regulated in development and DNA damage responses 1

TNFα –Tumor Necrosis Factor alpha

TNNT1- troponin T type1

TRPV1- transient receptor potential cation channel, subfamily V member 1

UPP- ubiquitin-proteasome pathway

Chapter 1: Introduction

1.1 Skeletal muscle and Atrophy

Skeletal muscle is the largest organ of the body and serves several important functions; from allowing locomotion through muscle contractions to regulating metabolism. It is a highly responsive tissue and is capable of adaptations to altered activity, metabolic stimuli, and pathological condition[1] . This includes muscle regeneration in response to an injury, the gaining of muscle mass in response to increased activity or growth stimuli, hypertrophy, or atrophy, the loss of skeletal muscle mass from lack mechanical stimuli during bed-rest, or in response to catabolic disease states, or aging[1,2]. Atrophy is the loss of skeletal muscle mass and function, through a decrease in muscle fiber size as a result of the loss of cytoplasm, organelles and proteins and it can result from malnutrition, immobilization and disuse, aging (sarcopenia) or diseases such as cancer, AIDS, chronic obstructive pulmonary disease (COPD) or chronic kidney disease[1,3]. Cancer cachexia is a type of atrophy and a complex multifactoral syndrome associated with cancer and is defined as the "involuntary loss of skeletal muscle mass, with or without the loss of fat mass, which cannot be reversed by nutritional interventions and leads to a severe impairment" [3,4].Increased skeletal muscle mass is strongly linked to success and tolerance of cancer treatments and therefore is the decrease in skeletal muscle mass directly linked to increased mortality and morbidity in cancer patients[5]. With cachexia affecting roughly 50% of all cancer patients, it is associated with ~20% of all cancer related deaths[6]. In refractory cachexia, the late stages of cachexia life expectancy is roughly three months[4,7,8]. Cachexia is also associated with anorexia, abnormal metabolism, negative protein balance and often, but not always systemic inflammation [4,5,8,9]. The loss of skeletal muscle mass and function associated with

aging, sarcopenia is multifactorial like cancer cachexia but includes factors such disuse, chronic inflammation, malnutrition as well as altered endocrine function[10]. Sarcopenia has also been associated with increased risks of falls, reduced independence and as in other atrophic conditions, with a decreased quality of life [7,11]. Some of the molecular mechanisms involved in these conditions differ, but all atrophic conditions ultimately stem from a disruption of the processes involved in the maintenance of skeletal muscle mass.

1.2 The maintenance of skeletal muscle mass by protein turnover, and changes in the turnover during atrophy

The maintenance of skeletal muscle mass and size is dependent on protein turnover, the balance between the protein degradation and the protein synthesis pathways within the muscle fibre. The processes involved in the degradation and synthesis of proteins are fluid and constantly ongoing, and altered according to the cellular needs and therefore allowing for the adaptation to changing conditions and stimuli[1,12]. Under normal conditions the cell maintains this balance between breakdown and synthesis. However when this balance is disrupted and either muscle protein synthesis is reduced, with no change in degradation rate, or the degradation of muscle proteins is increased, with or without a reduction in protein synthesis, atrophy occurs[2,12–14]. Many atrophy conditions share the same transcriptional program which leads to this change in turnover and negative protein balance[15]. There are two main pathways responsible for the degradation of proteins and organelles; the ubiquitin-proteasome pathway (UPP) and the autophagy pathway. Emerging evidence indicates a level of cross-talk between these pathways which allows for some compensation should the function of one pathway be

disrupted[16]. Under normal conditions these two protein degradation pathways protect the cell by the destruction of damaged, aged and misfolded proteins which otherwise could be harmful[12,17], but these pathways also allow the cell to undergo remodelling in response to physiological changes by the controlled breakdown of specifically selected proteins[13,18]. This enables the muscle cells to adapt to changes in the physiological conditions and in turn, adaptation of the skeletal muscle to changes in activity. Misregulation and disruption of the function of these pathways can lead to inadequate clearance and the build-up of misfolded, damaged or otherwise harmful proteins and this has in turn been shown to play a role in the several myopathies and muscular dystrophies[12,19,20]. While the functions of these two pathways are interlinked, the direct processes involved in the selection of a protein for a particular pathway and the following degradation of the proteins differ between the two.

The UPP use a multi-step pathway and poly-ubiquitin chains to target and remove short-lived sarcomeric proteins by degradation by the proteasome[16,18]. The multistep pathway starts with the activation of ubiquitin by ubiquitin-activating enzymes (E1) in an ATP-dependent fashion after which the activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2). An ubiquitin protein ligase (E3) then subsequently interacts with the E2 to identify the substrate and catalyses the attachment of ubiquitin to the target protein. Further E2-E3 interactions create a polyubiquitin chain which tags the protein for degradation by the proteasome. Only a protein with an attached polyubiquitin chain is recognised and docked onto the proteasome, which degrades the ubiquitinated protein into short peptides and amino acids which thereafter can be reused by the cell[1,21,22]. The ubiquitin tagging of selected proteins makes the proteasomal degradation of proteins a highly selective, but also reversible process as ubiquitin can be released by the deubiquiting enzymes (DUBs) although the process involved in

deubiquitionation are not fully understood[12,14,23]. A few ubiquitin ligases involved in this process have been characterized in skeletal muscles, and the two main ones are Muscle RING-finger protein 1 (MuRF1) and F-Box Protein 32 (FBXO32/MAFbx1/Atrogin-1). These E3 ligases are both involved in the ubiquitination of skeletal muscle proteins, and have been shown to be increased during atrophy[13,14]. MuRF1 ubiquitinates the myosin heavy chain and myosin light chain as well myosin binding protein C for UPP degradation[24]. While MuRF1 degrades myofibrillar proteins, FBXO32 targets the myoblast determination protein 1 (MyoD) and the eukaryotic translation initiation factor 3 subunit-F (eIF3-f). It has therefore been suggested that MuRF1 causes atrophy by directly degrading the myosin proteins and the thick filaments of the sarcomere. FBXO32 on the other hand has been shown to cause atrophy by down-regulating protein synthesis through its role as an E3 ligase for eIF3-f, and MyoD [25]. Knockout mice for MuRF1 or FBXO32 display a protection against muscle wasting by different stimuli and both E3 ligases have been found to be regulated by the activation of the members of Forkhead Box, subunit O transcription factors (FOXOs) [26]. By targeting myogenic factors increased FBXO32 activation leads to decrease protein synthesis[27].

Another muscle-specific F-box protein (FBXO40) has also been shown to negatively impact the protein synthesis mechanism by enhancing ubiquitination of the IRS1, insulin receptor substrate 1[28]. This increased proteasome-mediated degradation of IRS1 affects the IGF-1/IRS1/PI3K/Akt protein synthesis pathway and negatively affects muscle mass. Knockdown of Fbxo40 in C2C12 myotubes result in hypertrophy, increasing myotube size by as much as 50%[28]. Young Fbxo40 null mice also display a similar hypertrophic phenotype, being larger and having more skeletal muscle mass

compared to wild type mice[28]. The activity of these ubiquitin ligases therefore not only regulates the protein degradation process but also in part regulate the protein synthesis pathways as well. These observations clearly show an involvement of the ubiquitin proteasome pathway in atrophy.

The other main pathway involved in protein degradation is the autophagy-lysosome pathway, which controls the destruction of damaged and misfolded proteins, protein aggregates, as well as cytoplasm and organelles like mitochondria by delivering these to the lysosome[12,14,20]. Autophagy serves, just as the UPP, an important function in the recycling of proteins and amino acids, but also acts as a quality control mechanism of synthesised proteins and aids in cellular remodelling[14,18,20,21]. Because of these important functions in the maintenance of cellular functions, the malfunctioning of the autophagic system in muscle plays a large role in myopathies and malfunctioning of this process in other tissues than skeletal muscle has been linked to many human diseases such as Parkinson's disease and cancer[17,18,21]. There are three types of autophagy; macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is characterised by the formation of double-membrane vacuoles, autophagosomes, which are used for the delivery of proteins and material to the lysosome where degradation takes place. An autophagosome forms around a portion of the cytoplasm, including the protein aggregates, organelles and other materials that are to be degraded. Next the mature autophagosome fuses with the lysosome forming an autolysosome whereby the outer membrane of the autophagosome is incorporated into that of the lysosome and the inner autophagosome membrane is degraded together with the content of the vacuole[1,14]. Microautophagy on the other hand involves the direct engulfment of small portions of the cytoplasm by the lysosome without the formation of autophagosomes[20]. The third type of autophagy is chaperone-mediated autophagy, and

it requires the recognition of the KFERQ motif, a specific amino acid sequence, on the protein by the Heat shock cognate 70 (Hsc70) chaperone. The chaperone-mediated autophagy facilitates the degradation of damaged cytosolic proteins displaying this KFERQ-sequence, as the Hsc70 chaperone delivers these proteins to the lysosome via the lysosomal-associated membrane protein 2 (Lamp2a) receptors on the lysosomal membrane translocating the protein across the lysosomal membrane and into the organelle where it is finally degraded[1,20,29,30]. The use of ubiquitin molecules to tag proteins for degradation is also used by the autophagy-lysosome pathway and these are the mono-ubiquitinated proteins, and not the poly-ubiquitinated proteins targeted by the ubiquitin-proteasome pathway. When recognised, these mono-ubiquitin tagged proteins are then degraded by the lysosome. However further research is needed to fully elucidate the exact mechanisms involved in the recognition of these proteins by the lysosome and the selectivity for a particular pathway [13,14,30]. This however highlights some overlap in the processes involved in these two protein degradation pathways.

Because of its role in maintaining cellular function the autophagy-lysosome pathway is present at low levels under normal conditions maintaining muscle cell function, but is further activated in atrophy conditions[14]. Autophagy genes like microtubule-associated protein 1 light chain 3 (LC3), BCL2/adenovirus E1B 19kDa protein-interacting protein 3 (BNIP3) and gamma-aminobutyric acid receptor-associated protein-like 1 (GABARAPL1) are all up-regulated in atrophy[30]. LC3 and GABARAPL1 play an important role in the formation of the autophagosome membranes and the fusion with the lysosome and BNIP3 is one of the key regulators of mitochondrial degradation and biogenesis[31]. The activation of FOXOs has been shown to be necessary for the activation of these autophagy genes and for their role in atrophy. Inhibition of FOXO activity has been shown to prevent skeletal muscle atrophy and the up-regulation of

these genes[31–33]. Therefore the actions of LC3, GABARAPL1 and BNIP3 are under FOXO control just as MuRF1 and FBXO32[13,31–33]. FOXOs therefore control both of the protein degradation pathways and regulate the increased degradation seen in atrophic conditions, but also regulate the rate of protein synthesis through the Akt/mTOR pathway.

Protein synthesis is stimulated by feeding and skeletal muscle activity[34]. The main pathway of protein synthesis, and hypertrophy, is the insulin-like growth factor 1 (IGF-1)/ Akt/ mammalian target of rapamycin (mTOR) pathway. This signalling is mediated through the binding of IGF-1 to its plasma membrane receptor, the IGF1 receptor (IGF1R) and the resulting phosphorylation leads to the recruitment and interaction with IRS1 (insulin receptor substrate 1). The phosphorylation of IRS1 in turn leads to the down-stream phosphorylation of phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K), plasma membrane intrinsic protein 3 (PIP3) and the subsequent activation of Akt. Akt is a regulator of protein synthesis as it regulates the activity of the mammalian target of rapamycin (mTOR)[35]. mTOR exists as two complexes 1 and 2, mTORC1 and mTORC2, mTORC1 associates with RAPTOR and is inhibited by rapamycin. mTORC2 associates with RICTOR and upon activation acts as a positive feedback loop further stimulating the activation of Akt[34,35]. mTORC1 on the other hand is responsible for the activation of ribosomal protein S6 kinase, 70kDa, polypeptide 1 (p70S6K) and the eukaryotic translation initiation factor 4G (eIF4G) as well as the inhibition of the eukaryotic translation initiation factor 4E binding protein 1(4E-BP1)[13,36]. The inhibition of 4E-BP1 allows for the formation of the eukaryotic translation initiation factor 4F (eIF4F) complex which is required for the initiation of translation. The eIF4F complex is formed by the dissociation of eukaryotic translation initiation factor 4E (eIF4E) from

4E-BP1 and its subsequent binding to the phosphorylated eIF4G and eukaryotic translation initiation factor 4A (eIF4A), enabling the start of protein synthesis[25,34,37]. The activation of p70S6K and its downstream target ribosomal protein S6 regulates protein translation and leads to protein synthesis [13,25,37]. Akt not only stimulates protein synthesis through the activation of mTORC1, it also drives translation and protein synthesis by inhibiting GSK3β, thereby allowing the promotion of translation by the activity of eIF2B [13,25,34].

In addition to its role in the regulation of protein synthesis Akt also functions as an inhibitor of FOXOs and thereby regulates the protein degradation pathways[32,38]. Akt suppress the activity of the FOXO transcription factors preventing the transcription of MuRF1, FBXO32, BNIP3, GABARAPL1 and LC3, down-regulating both the ubiquitin-proteasome pathway and autophagy [14,32,38]. Latres et al (2005) showed that the atrophy in C2C12 myotubes induced by dexamethasone supplementation was reversed with the activation of the Akt/mTOR pathway by IGF-1 further supporting the role of the IGF-1/Akt/mTOR pathway in both protein synthesis and degradation. The inhibition of FOXO in mice and C2C12 myotubes also resulted in prevention of atrophy when models of Lewis lung carcinoma and sepsis were used[32]. In addition the knockout of S6K1, one of the downstream targets of the Akt/mTOR pathway, in myotubes resulted in an atrophy response emphasising the role of this pathway in the control of skeletal muscle protein turnover[39].

In summary the increased activation of the IGF-1/Akt/mTOR pathway leads to increased protein synthesis and increased skeletal muscle growth and hypertrophy in

skeletal muscle. The interplay between the levels of synthesis and degradation governs protein turnover and these processes also regulate each other. Protein synthesis has been shown to be suppressed in atrophy[40–43] while the protein degradation pathways are up-regulated [1,24,25,44].

1.3 The role of glucocorticoids and pro-inflammatory cytokines in atrophy

Glucocorticoids like dexamethasone have been frequently used to induce atrophy in rodents and cell culture. Excessive levels of glucocorticoids in patients suffering from Cushing's syndrome and in patients receiving glucocorticoid treatments cause skeletal muscle atrophy[15,45,46]. Dexamethasone treatments have been shown to induce skeletal muscle atrophy, reducing skeletal muscle mass, myofibre size, mitochondrial content and function in rodents and rodent cell lines[19,27,38,47–49]. Dexamethasone causes skeletal muscle atrophy by increasing protein degradation in combination with a down-regulation of protein synthesis. The activation of the glucocorticoid receptor (GR) in the plasma membrane by dexamethasone stimulates the up-regulation of regulated in development and DNA damage responses 1 (REDD1) and the transcription factor kruppel-like factor-15 (KLF-15)[48,50,51]. REDD1 is an mTOR inhibitor and while KLF-15 also blocks mTOR activation, more importantly it directly stimulates the expression of FBXO32 and the activation of FOXOs [25,52–54]. The inhibition or deletion of REDD1 in rodents or in L6 rat cells have been shown to prevent dexamethasone-induced atrophy [53–55] suggesting that the inhibition of protein synthesis might play an even more important role than the increased degradation in this form of atrophy. The activity of the GR therefore prevents skeletal muscle protein synthesis by the inhibition of mTOR

activity, and leads to an increased rate of degradation through the activity of both the ubiquitin-proteasome E3 ligases and autophagy.

Increased levels of pro-inflammatory cytokines have also been implicated in the induction of atrophy in cancer cachexia, sepsis and other wasting conditions[25,56]. The cytokine tumour necrosis factor-α (TNF-α) has been shown to be a useful marker for malnutrition in patients with gastric cancer[57] and to induce atrophy in cultured C2C12 myotubes[58]. TNF-α also inhibits myotube formation when administered to cultured human primary cells[59,60] and have been shown to up-regulate the expression of MuRF1 and FBXO32 via p38 mitogen-activated protein kinase (MAPK) and the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-$_K$B) pathway[14,56]. NF-$_K$B and p38 MAPK therefore drives atrophy through the increased degradation of proteins via the ubiquitin-proteasome pathway and targeted destruction of myosin heavy chain, myosin light chain and MyoD and eIF3-f by the increased expression of MuRF1 and FBXO32 as mentioned previously[14,25,56].

1.4 Capsaicin and the activation of TRPV1 as a potential treatment of muscle wasting

The pathways discussed above makes it clear that the changes in protein turnover associated with atrophy in wasting conditions are complex, and while the knowledge about the processes and pathways involved are growing, few interventions to date have been successful and there is currently no effective treatment to prevent or reverse this muscle wasting. However emerging evidence from rodent studies points towards a compound present in chilli peppers which seems to induce hypertrophy and protect against atrophy in mice through increased intracellular calcium levels $[Ca^{2+}]_i$.

Capsaicin, the transient receptor potential cation channel, subfamily V member 1 (TRPV1) agonist and the compound that gives chilli peppers their strong hot flavour, have been shown to have a protective effect against skeletal muscle atrophy in mice as well as regulate hypertrophy[61,62]. Ito et al (2013) found that neuronal nitric oxide synthase (nNOS) regulated overload-induced hypertrophy in mice by the production of nitric oxide and peroxynitrite leading to the activation the TRPV1, using a synergist ablation method where the plantaris muscle is forced to adapt and compensate for the gastrocnemius and soleus muscles after the ablation of the tendons to these synergistic muscles. Activation of the TRPV1 led to the release of $[Ca^{2+}]_i$ from the sarcoplasmic reticulum activating mTOR and stimulating skeletal muscle hypertrophy in these mice. Interestingly, when TRPV1 was activated by the administration of its agonist capsaicin, the effect on mTOR and p70S6K protein levels and phosphorylation was equivalent to the effects seen with overload. These changes were not seen in the TRPV1 null mice, suggesting that this hypertrophy response is indeed regulated by TRPV1 activation in mice skeletal muscle. They also found that mice receiving daily capsaicin supplementation resulted in increased mass of the soleus, plantaris and the gastrocnemius muscles, and increase in myofibre size, as well as increased muscle force production in the extensor digitorum longus (EDL) muscle, compared to mice who did not receive the supplement[61]. Leading on from these findings they investigated the potential for capsaicin-stimulated activation of TRPV1 to influence skeletal muscle during disuse atrophy. Interestingly mice supplemented with capsaicin during hindlimb suspension lost significantly less of the soleus and gastrocnemius muscles compared to control, and denervation resulted in a significantly larger loss of mass of the gastrocnemius muscle in control mice compared to the mice receiving the capsaicin treatment[61].

In another study following up on these results, the same group found that TRPV1 activation by capsaicin supplementation in mice resulted in increased phosphorylation of p70S6K and S6 without inducing phosphorylation of Akt, with similar changes being seen within 3 minutes of mechanical overload[62]. The findings from these studies suggest that capsaicin has the potential to induce skeletal muscle hypertrophy in a similar manner to resistance exercise and the potential to protect skeletal muscle mass from atrophy through the TRPV1-mediated increased levels of $[Ca^{2+}]_i$[61,62].

1.5 The problems involved with cell culture and rodent models of atrophy

As the majority of the research on atrophy have been conducted using rodent *in vitro* and *in vivo* models, with limited research being conducted using human cell culture or in clinical populations the evidence for the exact mechanisms taking place in humans during states of atrophy are not clearly understood. Similarly the research highlighted above suggesting that capsaicin acts as a resistance exercise mimic and has potential to protect against atrophy during denervation and hindlimb suspension needs further validating in human model systems before its role as a treatment against muscle wasting can be asserted. In addition previous research have shown that there are some slight differences in the response to dexamethasone when used as an atrophy-inducer in C2C12 mice skeletal muscle myotubes and L6 rat skeletal muscle myotubes[49]. Also, Owens et al (2013) reported differences in differentiation capacity of myoblasts and the response to treatment in myotubes, when human primary skeletal muscle cells obtained from different vendors were compared[63].

1.6 Aims and Objectives

In response to the issues highlighted above in section 1.5 the first aim of the present study was to investigate the differentiation capacity of the human primary skeletal muscle cells used and repeat the characterization of the gene expression during differentiation performed by Owens et al (2013) to locate the optimal time point during differentiation at which to perform our experiments. Secondly this study sought to investigate the effects of dexamethasone, TNF-α and capsaicin on these human skeletal muscle myotubes when administered to the culture in isolation to: i) investigate the effects of dexamethasone on these human skeletal muscle myotubes and validate the use of this glucocorticoid as a method to induce atrophy based on the findings of Owens et al, ii) to explore the suggested potential for capsaicin to induce hypertrophy in skeletal muscle using this human cell culture model, iii) also validate the use of TNF-α an atrophy-inducer in these cells as previous research indicates a role for TNF-α in atrophy, and iv) measure the potential changes in gene expression in response to these treatments. Lastly this study also aimed to further investigate and elucidate the potential for capsaicin to protect against dexamethasone-induced skeletal muscle atrophy.

Chapter 2: Materials and Methods

2.1 Cells culture

Human skeletal muscle myoblasts were purchased from Lonza (Clonetics® HSMM, Cat# CC-2580 Lot# 0000257384, Walkersville, USA). The cells were harvested from the quadriceps muscle of a non-diabetic 17 year old female donor. The myoblasts were cultured at 37°C with 5% CO_2, in growth medium (GM) consisting of Skeletal Muscle Cell Basal Medium without L-Glutamine (Clonetics®) supplemented with dexamethasone, Epidermal Growth Factor (EGF), 10% fetal calf serum, 2% L-Glutamine, gentamicin and amphoteracin. The cells were cultured to ~50-70% confluence. Prior to experiments cells were collected using Trypsin-EDTA (Gibco®) and counted using the TC20^{TM} automated cell counter (Bio-Rad Laboratories Inc.). To induce differentiation and start the experimental treatments cells were seeded in 6-well or 12-well polystyrene cell culture plates at various densities depending on the experiment and incubated overnight in GM. Differentiation was induced by transfer to differentiation medium (DM) consisting of Dulbecco's Modified Eagle Medium (2% horse serum, amphoteracin (250μg/ml) and gentamicin (10mg/ml) all from Life Technologies) following the recommendations by Lonza. Myoblasts were differentiated over 5-7 days with medium changed every 48 hours. Treatments were added to DM on day 3 or 5 when myotube formation was apparent microscopically.

2.2 Experimental design and treatments: The gene expression profile of differentiation

To investigate the gene expression profile of the human skeletal muscle myoblasts during the course of differentiation cells were seeded in 6 wells on four separate 12-well cell culture plates at ~$5.2x10^4$ cells per cm^2 and incubated in GM overnight. The following morning (Day 0) three wells from each plate were harvested into QIAzol® Reagent (Qiagen) and stored at -80°C for later analysis. The remaining cells were washed and DM added to the wells. Cells in DM were harvested at days 1, 3, 5 and 7 of differentiation into QIAzol reagent and stored at -80°C.

2.3 Experimental design and treatments: The effects of dexamethasone and capsaicin in isolation

Myoblasts were seeded at a density of ~$1x10^3$ cells per cm^2 in 6-well polystyrene cell culture plates. After 24 hours in GM cells were differentiated as above. On day 5 of differentiation DM was supplemented with either 50μM dexamethasone (Merck) or 100nM capsaicin (Merck), or with vehicle (DMSO) only. After 72 hours cells were harvested as above and stored at -80°C for later analysis.

2.4 Experimental design and treatments: The combined effects of dexamethasone and capsaicin and optimization of the capsaicin concentration

Myoblasts were seeded at $3.2x10^4$cells per cm^2 in 6-well plates in GM for 48 hours before being replaced with DM. After 72 hours the medium was changed to DM with DMSO (vehicle) or supplemented with dexamethasone at 50μM in DMSO, or a

combined treatment: 50μM dexamethasone plus capsaicin at 100nM, 1 μM, 10μM or 100μM all in DMSO. After a further 48 hours myotubes were harvested into QIAzol and frozen at -80°C for further analysis.

2.5 Experimental design and treatments: Investigating TNF-α induction of atrophy

Myoblasts were seeded at a density of $3.2x10^4$cells per cm^2 in 6-well plates in GM for 48 hours before being replaced with DM. After 72 hours DM was supplemented with TNF-α (Merck) at 10ng/ml, 20ng/ml or 40ng/ml in H_2O. After a further 48 hours cells were harvested as detailed above.

2.6 Fluorescence Microscopy

Cells were seeded in 6-well cell culture plate at ~$2.1x10^4$ cells per cm^2 and after 48 hours in GM nuclei were stained with 2.5μg/ml Hoechst 33342 dye (Invitrogen). Myotube nuclei, originally seeded at ~$5.3x10^4$ cells per cm^2 in GM, were stained with 2.5μg/ml Hoechst 33342 after 5 days in DM, and mitochondria were stained with 200nM MitoTracker Green (Life Technologies). Images were then obtained using a Zeiss Axiovert 135 microscope and Moticam Pro252B camera, and further processed using ImageJ (NIH) software.

2.7 RNA, DNA and Protein Isolation

RNA was extracted from the frozen cells by the addition of 200μl chloroform to the lysed cells in QIAzol. After a short incubation at room temperature the mixture was

spun at 12000g for 15 minutes. The upper aqueous phase was removed and the remainder set aside for DNA and protein extraction. 500µl isopropanol was added to the aqueous phase and incubated in room temperature for 30 minutes, after which the mixture was centrifuged at 12000g for 15 minutes to pellet the RNA. The pellet was washed in 75% ethanol, collected by centrifugation (7500g, 5 minutes) and air dried for 5 minutes at room temperature before being resuspended in 20µl RNAse free water. After RNA extraction DNA was isolated as follows. 300µl of 100% ethanol was added to residual material after RNA extraction. Samples were mixed by inversion, briefly incubated at room temperature and spun at 2000g for 5 minutes at 4^{o}C. The resulting supernatant (containing protein) was set aside at -80^{o}C. The DNA pellet was washed twice with 0.1M sodium citrate in 10% ethanol and then re-suspended in 1.5ml 75% ethanol. After a final spin at 2000g for 5 minutes the pellet was air dried and dissolved in 100µl 8mM NaOH. Insoluble material was removed by centrifugation at 12000g for 10 minutes. Protein was extracted from the remaining material by precipitation with 1.5ml isopropanol. After a 10 minute room temperature incubation protein was pelleted by centrifugation at 12000g for 10 minutes at 4^{o}C. The pellet was washed three times in 2ml 0.3M guanidine hydrochloride in 95% ethanol with a final wash in 2 ml 100% ethanol, with a 20 minutes incubation followed by centrifugation at 7500g for 5 minutes at 4^{o}C in between each wash. The protein pellet was collected by centrifugation at 7500g for 5 minutes and air dried for 20 minutes. The protein was re-dissolved in 150µl 1% SDS at 50^{o}C. Insoluble material was removed by centrifugation at 10000g for 10 minutes. DNA and RNA were measured using the Nanodrop 2000 (Thermo Scientific). Protein content was measured using the Biorad DC Protein assay (BioRad) with a standard curve prepared from bovine serum albumin ranging from 250µg/ml to 15.625µg/ml.

2.8 Gene Expression Analysis: qPCR

First strand cDNA was synthesised using the RevertAid First Strand synthesis kit (Thermo-Fisher) and oligo dT primers following the manufacturer's directions. 1µg of RNA was the input for each reaction. After mixing the kit components and RNA the reaction was heated at 42^{o}C for 60 minutes and then 70^{o}C for 10 minutes. First strand DNA was stored at -20^{o}C before further use.

qPCR was carried out using the Luminaris SYBR Green High ROX qPCR Master mix kit (Thermo-Fisher). The kit contents and cDNA were mixed according to the manufacturer's directions. Final primer concentration was 300nM for forward and reverse primers. Primer sequences used are shown in table 1. The PCR conditions were 50^{o}C for 2 minutes (UDG treatment), 95^{o}C for 10 minutes (DNA denaturation) followed by 40 cycles of 95^{o}C for 15 seconds and 60^{o}C for 60 seconds using the LightCycler 480 instrument (Roche). Primer efficiencies were calculated and are shown in table 2, and the raw qPCR data were analysed using the 2-delta-delta Ct method (Livak and Schmittgen, 2001). For all conditions, the gene expression levels were normalised to the expression of hypoxanthine phosphoribosyltransferase 1 (HPRT1). The expression of the structural proteins myosin heavy chain-2 (MYH2) and troponin T type1 (TNNT1), the transcription factors myogenin (MYOG) and myoblast determination protein 1 (MYOD1), as well as three genes involved in skeletal muscle remodelling: BCL2/adenovirus E1B 19kDa protein-interacting protein 3 (BNIP3), gamma-aminobutyric acid receptor-associated protein-like 1 (GABARAPL1) and F-box only protein 32 (FBXO32) were all measured by qPCR. All gene expression data were normalised and inverted, and are displayed in arbitrary units (AU).

Table 1. Forward and reverse primer sequences used for the qPCR analysis in this study.

Forward Primers	Reverse Primers
HPRT 5'-GCCAGACTTTGTTGGATTTG-3'	**HPRT** 5'-CTCTCATCTTAGGCTTTGTATTTTG-3'
BNIP3 5'-AACACGAGCGTCATGAAGAAAGGG-3'	**BNIP3** 5'-ATCCGATGGCCAGCAAATGAGAGA-3'
FBXO32 5'-GCCTTTGTGCCTACAACTGAA-3'	**FBXO32** 5'-CTGCCCTTTGTCTGACAGAAT-3'
GABARAPL1 5'-CCACCGCAAGGAGACAGAAG-3'	**GABARAPL1** 5'-GAAAATGTGATGACGGTGTGT-3'
MYOG 5'-GCTGTGAGAGCTGCATTCG-3'	**MYOG** 5'-CAGTGCCATCCAGTACATCH-3'
MYOD1 5'-CGCCATCCGCTATATCGAGG-3'	**MYOD1** 5'-CTGTAGTCCATCATGCCGTCG-3'
MYH2 5'-AGAAACTTCGCATGGACCTAGA-3'	**MYH2** 5'-CCAAGTGCCTGTTCATCTTCA-3'
TNNT1 5'-TGATCCCGCCAAAGATCCC-3'	**TNNT1** 5'-TCTTCCGCTGCTCGAAATGTA-3'

Table 2. The calculated primer efficiencies for the primers used for the qPCR analysis in this study.

Gene	Primer efficiency
HPRT	1.04
BNIP3	1.18
FBXO32	1.02
GABARAPL1	1.02
MYOG	1.19
MYOD1	1.03
MYH2	1.06
TNNT1	1.11

2.9 Statistical Analysis

All analysis was performed using Minitab® 17 Statistical Software (Minitab Inc.) and comparisons between groups were made using the One-Way ANOVA and Tukey's post-hoc analysis. Comparisons were considered significant when $p<0.05$. The gene expressions were normalised to the control group and displayed in the graphs below.

Chapter 3: Results

3.1 Characterisation of the human skeletal muscle myoblasts and differentiation into myotubes using fluorescence microscopy

Fluorescence microscopy was performed on myoblasts and differentiated myotubes as an additional measure to the gene expression analysis below to characterise the differentiation process. Figure 1 highlights the effects of the differentiation medium; by day 5 after the growth medium was exchanged for differentiation medium myoblasts have fused and formed multinucleated myotubes. There is a clear visual increase in stained, blue, nuclei after differentiation and these have aligned and fused into multinucleated myotubes (Fig.1A-B).

We also showed that it is possible to stain the mitochondria of these cells after differentiation using MitoTracker Green (Fig.1C). As atrophy is known to affect mitochondrial structures, number and function, this could be used to assess mitochondrial content and investigate the effects of dexamethasone and capsaicin on mitochondrial biogenesis.

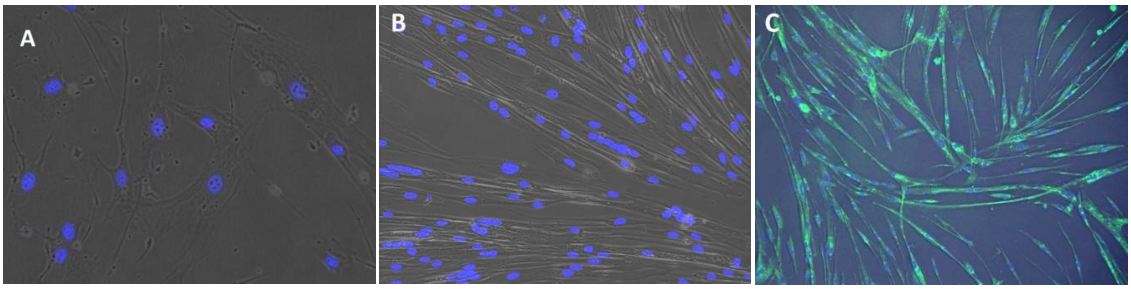

Figure 1. Microscopic images of the HSMM cells before and after induction of differentiation. A) Myoblasts kept in growth medium stained with Hoechst 33342 nuclei stain, nuclei in blue. B) Myotubes on day 5 of differentiation after the growth medium was exchanged for differentiation medium, nuclei stained blue using Hoechst 33342. C) Myotubes on day 5 of differentiation with nuclei stained blue using

Hoechst 33342 and mitochondria stained green using MitoTracker Green. All images taken using x20 magnification.

3.2 Characterisation of the gene expression profile of differentiation

We first examined the gene expression profiles of myogenic genes (MYOD1, MYOG and MYH2) across differentiation. Expression levels for these genes were measured at five different time points; before differentiation (GM) and at days 1, 3, 5 and 7 after the addition of differentiation medium (DM) (Fig. 2).

The expression of MYOD1 was not altered over the course of differentiation (Fig. 2a). There was however a significant increase in the expression of MYOG (Fig. 2b) from day 3 of differentiation and expression levels on day 5 were significantly higher than those of GM and day 1, yet this was not significantly different from day 3 and day 7. A similar response was seen for the expression of MYH2 (Fig. 2c) with a significant increase in expression from day 3 and onwards after the induction of differentiation compared to GM ($p=0.001$).

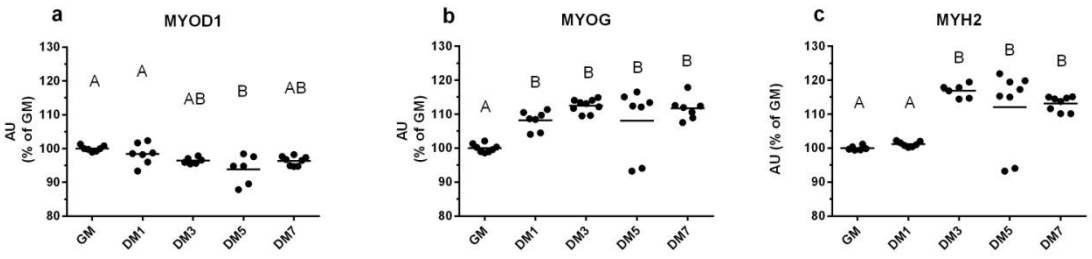

Figure 2. The expression of (a) MYOD1, (b) MYOG, and (c) MYH2 were measured before and over a 7 day period after differentiation. Conditions marked with different letters are significantly different from each other ($p<0.05$). GM= Cells in growth medium before differentiation, DM1= Cells on day 1 after medium was changed to differentiation medium (DM), DM3= Day three of differentiation, DM5= Day five of differentiation, DM7= Day seven of differentiation.

These results gave us an indication of the ideal time point during differentiation to examine the effects of dexamethasone and capsaicin individually on the myotubes. As the largest differences in gene expression compared to pre-differentiation levels were seen after day 3 of differentiation, day 3-5 were chosen as the time point at which the treatments were to be added. Microscopic examination of myotube formation ultimately finalised the exact time point for each of the experiments as differentiation was slower in cells cultures with a higher passage number and lower seeding density.

3.3 Characterisation of the gene expression response to Dexamethasone and Capsaicin

After 5 days of differentiation, when myotubes were apparent, the DM culture medium was treated with DMSO only, dexamethasone (50μM) in DMSO or capsaicin (100nM) in DMSO. The cells were harvested after a further 72 hours in their respective treatment. We examined the expression of myogenic and remodelling genes and also assessed the changes in DNA and protein content as well as the protein:DNA ratio as an indicator of myotube protein turnover.

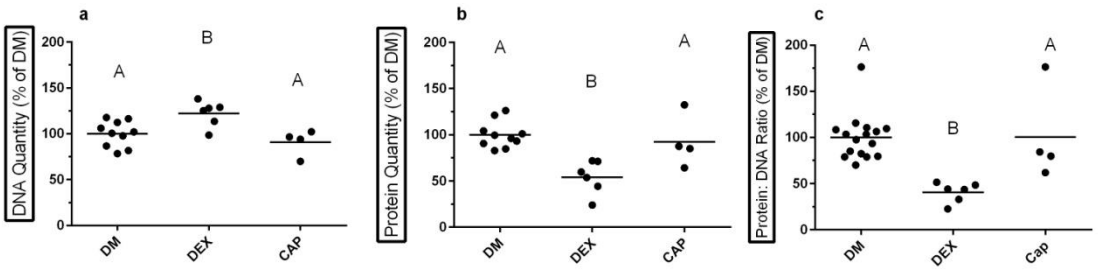

Figure 3. The (a) DNA and (b) protein quantities and (c) Protein:DNA ratio of the cells measured under the three conditions, DM= Control cells in differentiation medium+ DMSO, DEX= dexamethasone

treated cells, Cap= cells treated with 100nM capsaicin for 72 hours. Conditions that do not share a letter are significantly different from each other ($p<0.05$).

As shown in figure 3a the dexamethasone treatment significantly increased the DNA content compared to untreated (DMSO only) control cells ($p=0.019$) and to that of the cells receiving the capsaicin treatment ($p=0.008$). On the other hand the dexamethasone treatment significantly lowered the measured quantity of protein compared to that seen in the untreated control cells ($p=0.001$) and the capsaicin treated cells ($p=0.015$) (fig.3b). The protein:DNA ratio displayed the same pattern (fig.3c), with dexamethasone treatment resulting in significantly lower ratio compared to the untreated control cells ($p=0.001$) and cells receiving the capsaicin treatment ($p=0.007$). Capsaicin on the other hand did not significantly affect DNA and protein content compared to the DM cells nor did it affect the protein:DNA ratio. This shows that the capsaicin treatment had no adverse effects on myotube DNA and protein content during differentiation but a 50μM concentration of dexamethasone induced atrophy.

To examine whether the treatments had any effect on gene expression during differentiation we examined the expression of the structural proteins MYH2 and TNNT1 (Fig. 4), the myogenic transcription factors MYOG and MYOD1 (Fig. 5), as well as three genes linked to skeletal muscle remodelling BNIP3, GABARAPL1 and FBXO32 (Fig. 6).

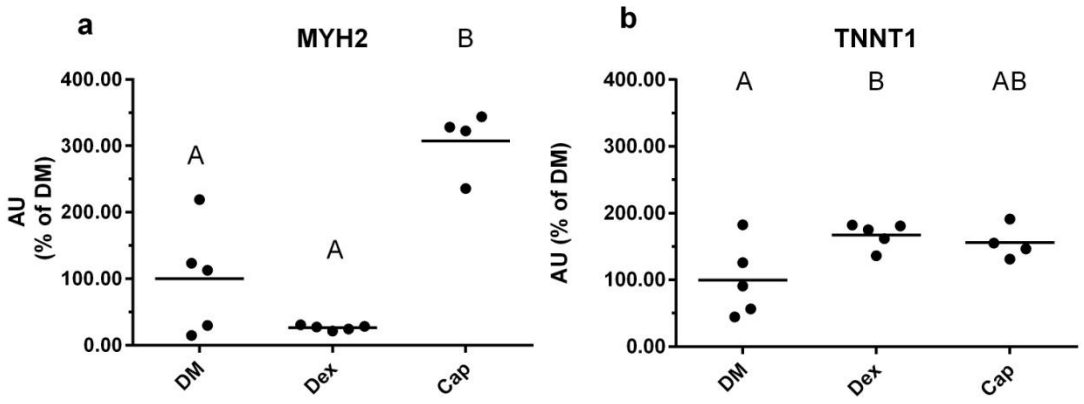

Figure 4. The relative gene expression of (a) MYH2 and (b) TNNT1 for each of the three conditions, DM=Untreated control cells (DMSO only), DEX=dexamethasone treated cells, Cap= cells treated with 100nM capsaicin. Conditions that do not share a letter are significantly different from each other ($p<0.05$).

As seen in Fig. 4a the expression of MYH2 was significantly elevated in the cells which received the 100nM capsaicin treatment both compared to the control cells ($p=0.001$) and the cells treated with dexamethasone ($p=0.001$). Although the mean MYH2 level was lower in the dexamethasone treated cells than the controls this did not reach statistical significance. Dexamethasone treatments significantly increased the expression of TNNT1 above that of the untreated control cells ($p=0.043$) but there was no significant change in expression with capsaicin treatment compared to the other two groups.

Next we measured effect of the treatments on the gene expression for the transcription factors MYOD1 and MYOG (Fig. 5). Dexamethasone treatment significantly increased the expression of MYOD1 compared to the cells in differentiation medium ($p=0.001$) and compared to the capsaicin condition ($p=0.032$). There was also a trend for increased

expression of MYOD1 with capsaicin treatment compared to control cells (p=0.05). For MYOG, capsaicin treatment significantly elevated the expression above that of both untreated control (p=0.004) and dexamethasone treated cells (p=0.007) (Fig. 5b). Expression in the dexamethasone treated group did not differ from that of the untreated control cells.

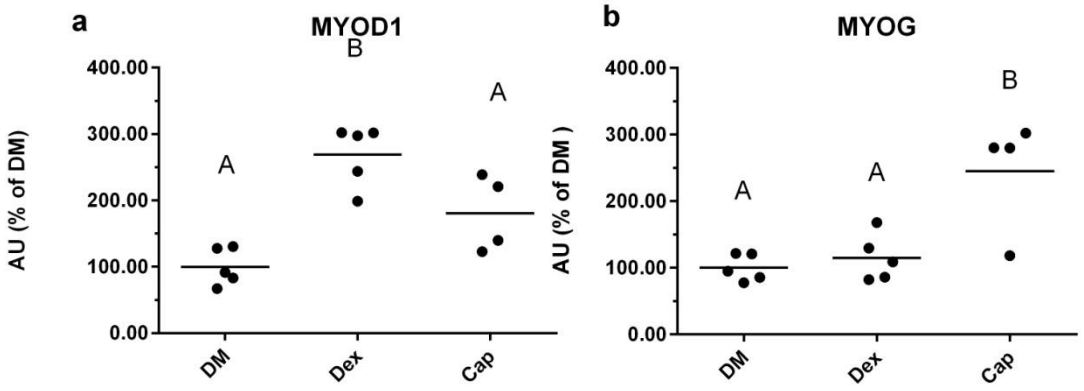

Figure 5. The relative gene expression of the transcription factors (a) MYOD1 and (b) MYOG for each of the three conditions. Conditions that do not share a letter are significantly different from each other ($p<0.05$).

Neither capsaicin nor dexamethasone treatment had any effect on the expression of the remodelling genes BNIP3 and FBXO32 (Fig. 6a, c), however capsaicin significantly elevated the expression of GABARAPL1 compared to both untreated control (p=0.002) and dexamethasone (p=0.023) (Fig. 6b). The dexamethasone treatment did not induce in any changes in GABARAPL1 expression compared to control cells.

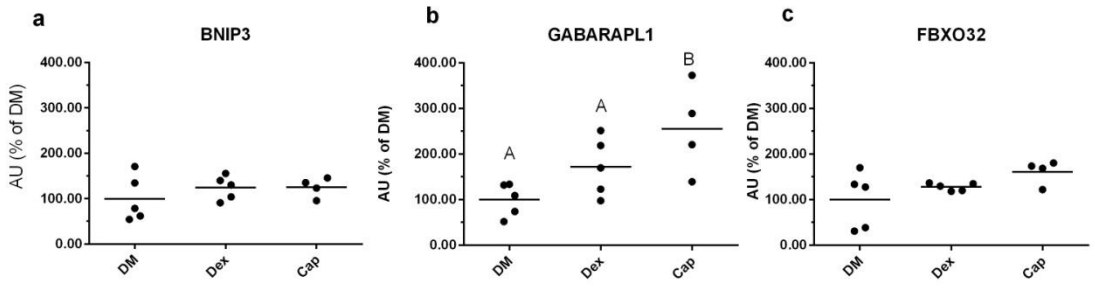

Figure 6. The expression of the three remodelling genes (a) BNIP3, (b) GABARAPL1 and (c) FBXO32 for each of the three conditions. Conditions that do not share a letter are significantly different from each other ($p<0.05$).

3.4 Optimisation of Capsaicin concentration and the combined effect of Dexamethasone and Capsaicin on gene expression

From these results using the 100nM capsaicin solution we learned that this concentration was a well tolerated by the cells and induced measurable changes in the gene expression of the skeletal muscle myotubes without negatively affecting the cellular protein content. In order to test whether capsaicin was capable of reversing the loss in protein seen in dexamethasone induced atrophy we designed a dose response experiment. After 3 days in differentiation medium, cells were left untreated (DM+ DMSO only) or treated with dexamethasone only at 50µM or dexamethasone at 50µM plus capsaicin at 100nM, 1µM, 10µM or 100µM all in DMSO. Results for the protein:DNA ratio analysis are shown in figure 7.

While none of the capsaicin concentrations altered the protein content (fig.7b) and protein:DNA ratio (fig.7c) compared to that found in the untreated cells, both the ratio and the protein content of the cells treated with dexamethasone and 100nM capsaicin

was significantly higher than that of the cells treated with dexamethasone-only (protein content p=0.044, ratio p=0.008) (Fig. 7). Conversely the dexamethasone only treatment resulted in a small non-significant reduction in protein content and protein:DNA ratio compared to control. At capsaicin concentrations greater than 100nM there was no effect of the combined dexamethasone-capsaicin treatments compared to dexamethasone alone. DNA content was not changed with any of the treatments used (fig. 7a).

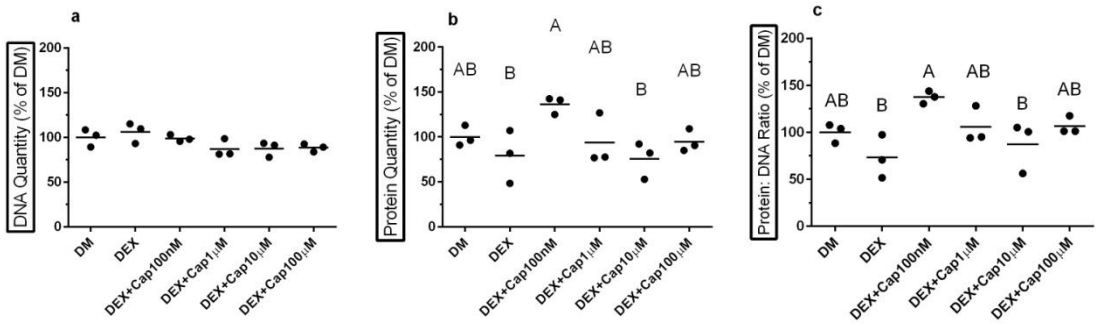

Figure 7. The (a) DNA and (b) protein quantities and (c) Protein:DNA ratio of the cells measured under the six conditions of the dose-response experiment, DM= Untreated control, DEX= 50μM dexamethasone only, DEX+Cap100nM= 50μM dexamethasone and 100nM capsaicin, DEX+Cap1μM=50μM dexamethasone and 1μM capsaicin, DEX+Cap10μM= 50μM dexamethasone and 10μM capsaicin, DEX+Cap100μM= 50μM dexamethasone and 100μM capsaicin . Conditions that do not share a letter are significantly different from each other ($p<0.05$).

In addition while no significant changes to the quantity of DNA and protein, and to the protein:DNA ratio were seen with the 100μM treatment, these cells underwent a gross phenotypic change and detached from the polystyrene tissue culture wells (not shown).

From the protein content measurement we concluded that the 100nM concentration of capsaicin was the most effective concentration for relieving dexamethasone induced myotube atrophy and we repeated the above experiment using only 100nM capsaicin in combination with dexamethasone to profile gene expression changes. Thus we measured the expression of MYH2, TNNT1, MYOG, MYOD1, BNIP3, GABARAPL1 and FBXO32 in untreated myotubes or myotubes treated with 50μM dexamethasone or 50μM dexamethasone+100nM capsaicin (Fig. 8-10).

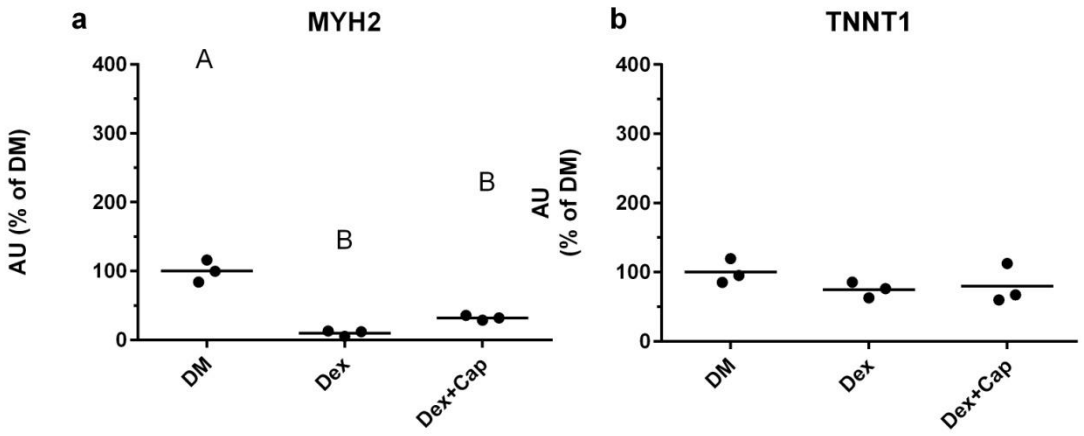

Figure 8. The expression of (a) MYH2 and (b) TNNT1 for the three conditions, DM=Untreated control cells, Dex=dexamethasone treated cells, Dex+Cap= cells treated with 50μM dexamethasone and 100nM capsaicin. Conditions that do not share a letter are significantly different from each other ($p<0.05$).

The expression of MYH2 was dramatically decreased with the dexamethasone treatment as seen in figure 8a ($p=0.001$). There was a slight rise in MYH2 expression when dexamethasone and capsaicin were combined but this was not significant. There were no effects of treatment on TNNT1 expression.

MYOD1 expression (Fig. 9a) was significantly increased above the levels of the control cells with dexamethasone treatment (p=0.002), and although the combined treatment seemed to inhibit that increase the difference did not reach statistical significance. There was no effect on MYOG expression (Fig. 9b).

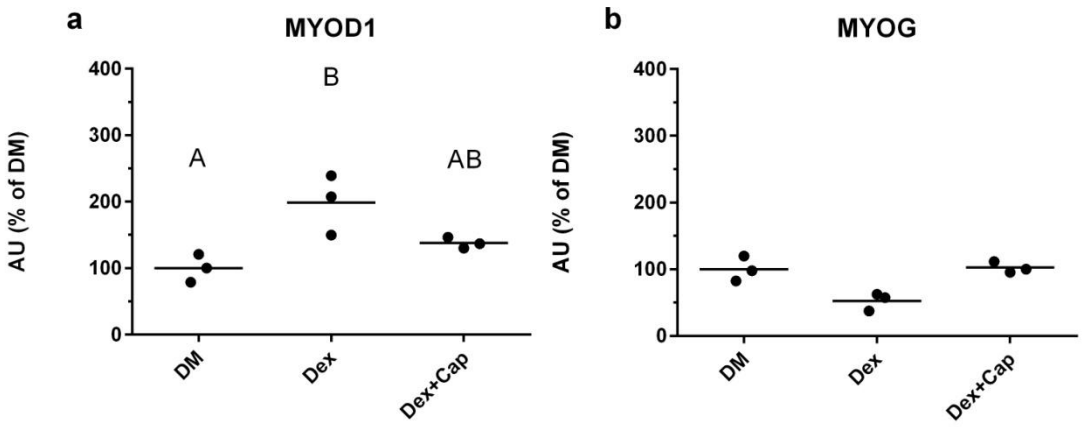

Figure 9. The expression of the myogenic transcription factors (a) MYOD1 and (b) MYOG from the three treatments, DM= untreated control cells, Dex=dexamethasone treated cells, Dex+Cap= cells treated with 50μM dexamethasone and 100nM capsaicin. Conditions that do not share a letter are significantly different from each other (p=<0.05).

The expression of the remodelling genes BNIP3, GABARAPL1 and FBXO32 for each of the conditions are shown in figure 10. Neither the dexamethasone treatment alone nor the combined dexamethasone+100nM capsaicin affected the expression of these genes.

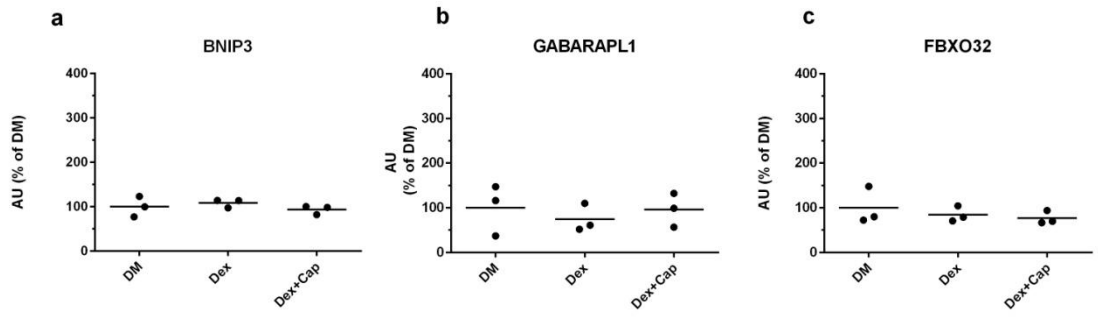

Figure 10. The expression of the remodelling genes (a) BNIP3, (b) GABARAPL1 and (c) FBXO32 for the three conditions, DM= untreated control cells, Dex=dexamethasone treated cells, Dex+Cap= cells treated with 50μM dexamethasone and 100nM capsaicin.

3.5 Validation of TNF-α as an inducer of atrophy in human primary cells

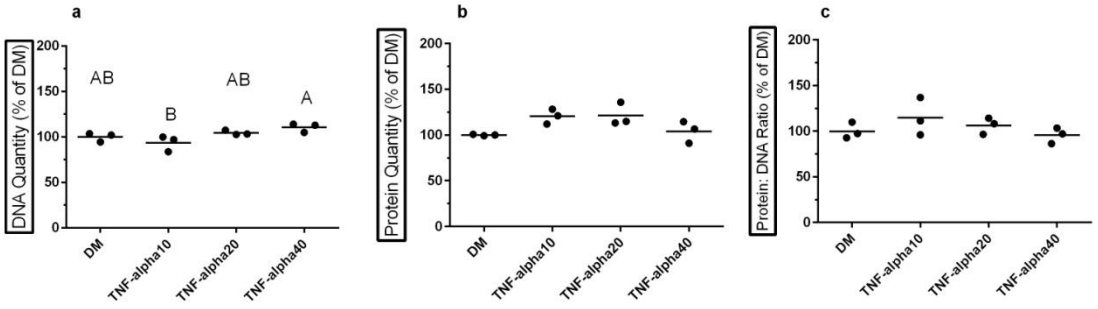

Figure 11. The (a) DNA and (b) protein quantities and (c) Protein:DNA ratio measured for the untreated control and the cells treated with the three different concentrations of TNF-α (TNF-alpha10 = 10 ng/ml, TNF-alpha20= 20ng/ml and TNF-alpha40= 40 ng/ml). Conditions that do not share a letter are significantly different from each other ($p<0.05$).

Lastly we wanted to do a similar dose response experiment for TNF-α as this cytokine has previously been used to induce atrophy in other *in vitro* skeletal muscle models. We first used a concentration of 5ng/ml of TNF-α (data not shown) and found no effect on

protein content or expression of the genes investigated. We therefore wanted to explore the effects of higher concentrations of TNF-α on the human skeletal muscle myotubes. The concentrations chosen were 10ng/ml, 20ng/ml and 40ng/ml of TNF-α, and the protein content for each concentration is displayed in figure 11.

As shown in figure 11a the different doses of TNF-α had no effect on the DNA content compared to the untreated control cells, however there was a significant increase with the 40ng/ml concentration compared to the lower 10ng/ml TNF-α concentration (p=0.027). Protein content (fig.11b) and protein:DNA ratio (fig.11c) in these human cells were not changed in response to any of these TNF-α treatments. While there is a small downward trend with increasing concentration there were no significant differences between any of the treatment conditions.

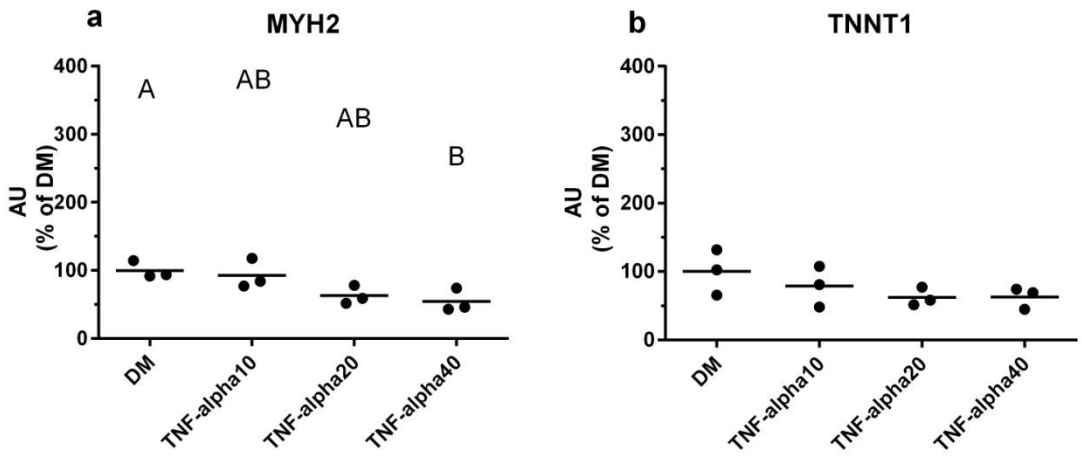

Figure 12. The expression of (a) MYH2 and (b) TNNT1. DM= untreated control, TNF-alpha10 = 10 ng/ml TNF-α, TNF-alpha20= 20ng/ml TNF-αand TNF-alpha40= 40 ng/ml TNF-α. Conditions that do not share a letter are significantly different from each other ($p<0.05$).

Despite not showing an effect on the protein content, the expression of the seven genes tested previously in the dexamethasone and capsaicin experiments were measured for each of the three concentrations of TNF-α. While there is a general decline in expression with increasing TNF-α concentration, only the reduction in MYH2 expression induced by the 40ng/ml concentration reached significance (Fig. 12a) compared to the untreated control ($p=0.042$). The expression seen with this concentration was not significantly lower than the other three concentrations investigated.

TNF-α concentration had no effect on TNNT1 expression (Fig. 12b) and no changes were seen with the different concentrations on the expression of MYOD1, MYOG and the remodelling genes BNIP3, GABARAPL1 and FBXO32 (Fig. 13a-e)

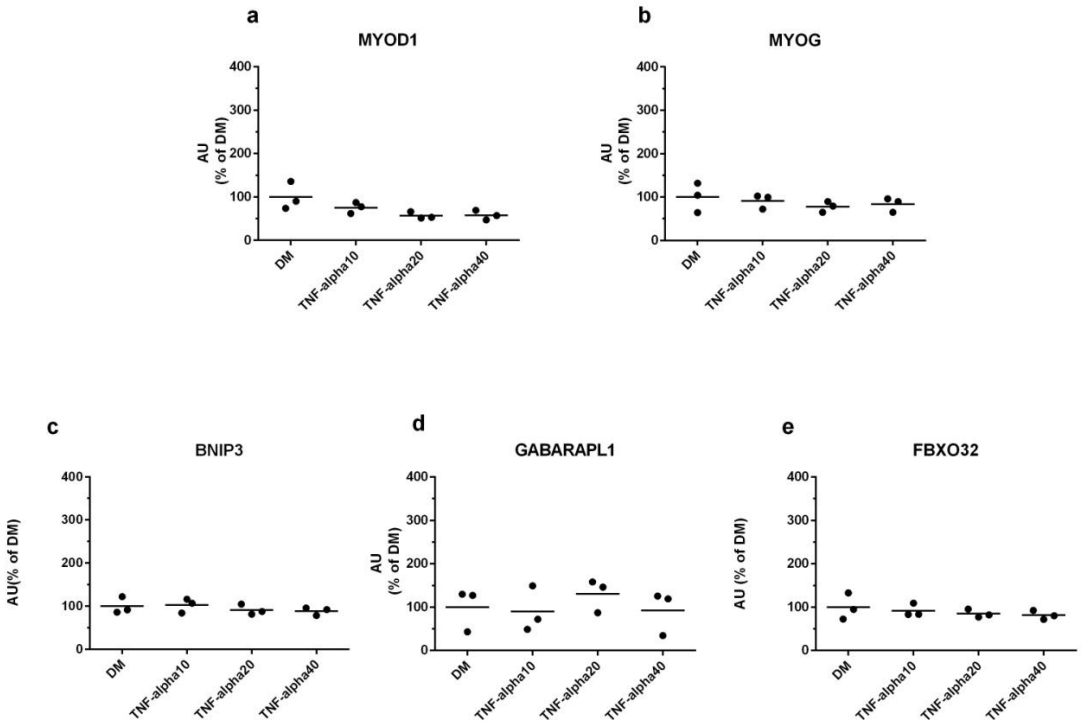

Figure 13. The expression of the transcription factors (a) MYOG and (b) MYOD1as well as (c) BNIP3, (d) GABARAPL1 and (e) FBXO32 which are linked to skeletal muscle remodelling, of the cells after receiving one of four treatments. DM= untreated control, TNF-alpha10 = 10 ng/ml TNF-α, TNF-alpha20= 20ng/ml TNF-α and TNF-alpha40= 40 ng/ml TNF-α.

Chapter 4: Discussion

In the present study human skeletal myoblasts were used to asses and validate the use of dexamethasone and TNFα as methods to induce skeletal muscle atrophy in this commercially available cell line after differentiation into myotubes. The study also sought to elucidate the effects of capsaicin and the cellular response to treatments with dexamethasone, dexamethasone together with capsaicin, capsaicin alone and TNF-α by measuring DNA and protein contents, protein:DNA ratio and gene expression. In addition, to also investigate the potential protective effects of capsaicin against dexamethasone-induced skeletal muscle atrophy. As Owens et al (2013) found some differences in the differentiation capacity and the response to treatments in human skeletal muscle cell lines from different vendors, the first aim of this study was therefore to characterise the differentiation process in these skeletal muscle cells. From the gene expression assessment of MYOD1, MYOG and MYH2 differentiation was confirmed in agreement with the findings by Owens et al (2013) and the information supplied by Lonza, validating the use of this cell line as a model for investigating skeletal muscle cell adaptation.

This study shows that 50μM of dexamethasone is sufficient to induce atrophy in these myotubes as treatment significantly reduced the protein:DNA ratio and MYH2 expression, without inducing any changes in the expression of genes associated with protein degradation. 100nM capsaicin when added to the DM caused no toxic effects and induced significant increases in the expression of MYH2 and MYOG, as well as GABARAPL1. The combined treatment of dexamethasone and capsaicin did not display the same level of changes in gene expression as the dexamethasone-only

treatment. This may indicate that capsaicin has the potential to ameliorate some of the effects of dexamethasone and somewhat normalize gene expression, brining it closer to the levels seen in the untreated control cells. TNF-α treatment with a range of different concentrations failed to induce atrophy in these myotubes.

Skeletal muscle mass is maintained through the careful control of protein turnover, the balance between the degradation and synthesis of proteins. Protein degradation is mediated through two main processes; the ubiquitin-proteasome pathway and autophagy. Atrophy is the loss in skeletal muscle mass and decrease in muscle fibre size as a result of a disruption in the control of these two processes[1,3,12,15,25]. There are several causes for atrophy it can be the result of malnutrition, aging and diseases like cancer, AIDS and COPD[7,10,13]. Skeletal muscle mass is in many of these conditions directly linked to treatment success, quality of life and mortality. In cancer wasting of skeletal muscle is seen in roughly 50% of all cancer patients and it has been associated with ~20% of all cancer related deaths[4,7]. The age associated loss of skeletal muscle mass, sarcopenia, in turn has been linked to increased risks of falls, frailty and reduced quality of life[10]. While the conditions causing atrophy do so through slightly different mechanisms, all atrophic conditions ultimately stem from a disruption in the processes maintaining skeletal muscle mass[15].

Skeletal muscle is a responsive tissue capable of adaptation in response to stimuli and regeneration when injured. This generation of new skeletal muscle is termed myogenesis[64]. Myogenesis allow the skeletal muscle to balance the turnover of skeletal muscle cells, and for active renewal to take place. Myogenesis in adult skeletal muscle

is dependent on the activation of satellite cells and their differentiation into new myotubes and myofibers, and this, is in part, regulated by the expression of myogenic regulatory factors (MRFs)[64,65]. The activation of satellite cells by these myogenic regulatory factors gives rise to progenitor cells committed to the myogenic program, myoblasts, which undergo differentiation to form myotubes. The myogenic factor 5 (Myf5) and MyoD commits the myoblasts to the myogenic program and starts the differentiation process[64]. The expression of MyoD is increased in the beginning of the differentiation process, but in the later stages of differentiation this is reduced with a coordinated increase in the MFRs myogenin and myogenic regulatory factor 4 (MRF4). Myogenin and MRF4 facilitate the fusion of differentiating myoblasts into myotubes and terminate the differentiation process [64–66]. Owens et al (2013) investigated the differentiation capacity of four human primary skeletal muscle cell lines and characterized the expression of MYOD and MYOG and other markers of cellular differentiation, TNNT and MYH2, and found differences both in capacity and expression of these markers in different muscle cell lines[63]. Therefore this study first sought to characterise the expression of MYOD, MYOG and MYH2 in the skeletal muscle cells used. While the expressions of MYOG and MYH2 are in agreement with the expression levels observed by Owens et al (2013) significantly increasing on day 3 after differentiation was induced this was not the case with MYOD. MYOD expression was expected to be significantly elevated by day 1 of differentiation and to afterwards decrease as expression of MYOG and MYH2 increases. In this study however, there is a small, non-significant increase in MYOD expression on day 1 of differentiation. This discrepancy with previous literature is likely due to the high variability of the data, which is very clear in the data of day 5 (fig.2), and it is probable that this masks some of the effects of differentiation. With this small increase in expression of MYOD seen on

day 1 taken into account with the elevations of MYOG and MYH2 expression on day 3, then expected expression pattern of these MRFs during differentiation were confirmed in this skeletal muscle cell culture model.

When the balance in protein turnover is disrupted and the muscle enters a state of negative balance as degradation rates exceed the rates of synthesis, atrophy occurs. There are two main degradation pathways which both play a role in the maintenance of cellular function and development, the UPP and the autophagy pathway and both have been shown to be up-regulated during atrophy. UPP targets proteins for degradation using a three step pathway where the interaction of an E1-activating enzyme, an E2-conjugating enzyme and an E3 ligating enzyme tags proteins with chains of poly-ubiquitin molecules enabling the proteasome to degrade the specific protein [1,13,27]. The autophagic pathway consists of three different mechanisms for the degradation of proteins and organelles by the lysosome; microautophagy involves the direct engulfment of parts of the cytosol by the lysosome, chaperone-mediated autophagy requires the recognition of a specific amino acid sequence by Hsc70 for the translocation of the protein into the lysosome to take place, and macroautophagy involves the formation of a double membrane vacuole, an autophagosome, containing the protein, or organelle which is to be degraded. The autophagosome then fuses with the lysosomal membrane, enabling the degradation of the inner membrane of the autophagosome and its content by the lysosome[13,14,30].

Glucocorticoids like dexamethasone have been frequently used to induce atrophy in rodents and cell cultures as excessive levels of glucocorticoids in patients suffering

from Cushing's syndrome and in patients receiving glucocorticoid treatments cause skeletal muscle atrophy[15,45,46]. Dexamethasone treatments have been shown to reduce skeletal muscle mass, myofibre size, mitochondrial content and function in rodents and in mouse C2C12, rat L6 cell lines[19,27,38,47–50,67] and human skeletal muscle cell lines[63]. Dexamethasone, and other glucocorticoids, induces atrophy by altering both protein breakdown and synthesis. By activating the glucocorticoid receptor and its target genes REDD1 and KLF15, protein synthesis via the Akt/mTOR pathway is inhibited and degradation is stimulated through the action of KLF15 on FBXO32 and FOXO[14,30,31,33,53]. As FOXO-activation is known to drive the up-regulation of the E3 ligases MuRF1 and FBXO32, and facilitate their translocation into the cytoplasm, this increases the degradation of their substrates MyoD, eIF3-f, myosin heavy chain and myosin light chain through the UPP[24,26,33]. Dexamethasone also increases the activation of the autophagy pathway through the activation of FOXO[50,52], as FOXO in turn governs the expression of LC3, BNIP3 and GABARAPL1 which play a key role in the formation of the autophagosome and the targeted degradation of mitochondria[14,30,31]. In addition, dexamethasone treatment have also been shown to inhibit both the phosphorylation of Akt and Akt protein content in skeletal muscle, and due to its role in mTOR signalling and activation, this have direct effects on protein synthesis. In addition, mTOR as well as the phosphorylation of the downstream target p70S6K have been shown to be inhibited by dexamethasone treatment[1,26,48,50,51,68]. As Akt also functions as an inhibitor of FOXO, the dexamethasone blockage of Akt therefore acts on FOXO in two ways. The down-regulation of the protein synthesis pathways and the up-regulation of protein degradation through both the UPP and the autophagy pathway lead to the reduced muscle fibre size, disruption of myotube formation and decrease in myotube size seen in dexamethasone-induced atrophy[38,48,49,52].

Previous studies have highlighted differences in the response to dexamethasone between cell lines of different species[49], and some differences between commercially available human skeletal muscle cell lines as well[63]. Thus, it was important to firstly validate the use of dexamethasone and the 50μM concentration in this line of muscle cells. The results of the present study show that dexamethasone treatment did indeed induce some of the expected changes in protein turnover and gene expression. The addition of dexamethasone to the DM reduced MYH2 expression and greatly reduced the protein: DNA ratio in these cells. Expression of TNNT1 differed between the first and the second experiment and as this is likely due to the differences in treatment length and the stage of differentiation at which the dexamethasone was added, it is impossible to draw any conclusions regarding the effect of this treatment on TNNT1 expression. Surprisingly, this dexamethasone treatment did not induce any changes in the expression of MYOG, BNIP3, GABARAPL1 and FBXO32 contrary to previous studies. The treatment also resulted in a large increase of MYOD1 expression, which was also somewhat unexpected. MYOD1 has been shown previously to be down-regulated with dexamethasone treatment and the protein being a substrate for FBXO32[24,27]. However protein levels of FBXO32 and MYOD1 were not measured in this study and the expression of FBXO32 was unaffected by dexamethasone treatment, therefore it is likely that there is an alternative explanation for this increase in MYOD1. However, there are some studies that support this finding. MYOD expression has also been shown to increase with dexamethasone treatment in C2C12 myoblast[69], male rat anterior tibialis muscle[70] but also in the skeletal muscle of chickens[71]. Even more interesting to this is the finding by Pessina et al (2010). They found that MYOD expression is increased in male and female patients with gastric cancer, but only in the early stages of the disease[72]. Together these findings may indicate a cellular response to

the atrophy whereby MYOD is part of a regeneration process to counteract the atrophy and increased protein degradation. While some of these findings are somewhat different from previous research, based on the protein content and protein: DNA ratio data and the reduction in MYH2, dexamethasone successfully induced atrophy in these human skeletal myoblast, and while no further up-regulation of the ubiquitin-proteasomal or autophagic-lysosomal pathways were seen, these are ongoing processes in differentiating cells and are likely to still play a vital role in the atrophy response seen in the current study.

Ito et al (2013) showed that nNOS regulated overload-induced hypertrophy in mice by the production of nitric oxide and peroxynitrite, leading to the activation the TRPV1. They did this using synergistic ablation of the tendons to the soleus and gastrocnemius muscles to stimulate overload-induced hypertrophy of the plantaris muscles. The resulting increase in $[Ca^{2+}]_i$ by the activation of TRPV1, phosphorylates mTOR and the down-stream targets p70S6K and S6 without the activation of Akt[61]. Interestingly these studies also showed that dietary supplementation of the TRPV1 agonist capsaicin resulted in similar phosphorylation patterns to when TRPV1 was stimulated by nitric oxide and peroxynitrite, in addition mice receiving only the capsaicin supplement displayed a hypertrophy phenotype compared to the control group[61]. The follow-up experiment to this found that dietary supplementation of capsaicin prevented some of the muscle loss normally seen during denervation and hindlimb suspension in mice through the activation of TRPV1 and its subsequent activation of the mTOR pathway. They proposed capsaicin to be a mimic of resistance exercise as the phosphorylation pattern induced by capsaicin supplementation in these mice highly resembled the pattern induced by their model of mechanical overload[62]. As resistance exercise is one

of the few successful ways to stimulate mTOR activation and to induce hypertrophy[35] the suggested potential of capsaicin to mimic these responses and to prevent atrophy offers great possibilities.

As these studies were performed in mice we therefore wanted to investigate the effects of capsaicin on human muscle and therefore administered capsaicin in isolation as well as in combination with atrophy-inducing dexamethasone to myotubes differentiated from a human skeletal muscle cell line. When 100nM capsaicin was administered to the myotubes in isolation there were no changes in either DNA content, protein content or protein:DNA ratio, and it is therefore concluded that capsaicin at this concentration has no toxic effects. From this observation it is also unlikely that capsaicin induces any large-scale effects on protein turnover. However the addition of capsaicin to the medium resulted in a large increase in the expression of MYH2. This would be consistent with the increases in muscle mass found by Ito et al (2013). Thus, if capsaicin truly were a resistance exercise mimic then its action would lead to larger fibres and an increased need for myosin heavy chain proteins[35,61,62]. However increased concentrations of capsaicin offered no additional effect in our cells. The current study, and the Ito studies did not measure myosin heavy chain protein levels, neither was the levels of the ubiquitin ligase MuRF1 which is known to degrade myosin heavy chain, measured. Therefore to fully understand the process and the implication of the increased MYH2 expression, these measures would be necessary. Capsaicin treatment also increased MYOG and GABARAPL1 expression, indicating an increased rate of differentiation and fusion into myotubes as the transcription factor myogenin is expressed later in the differentiation process and known to govern the termination of differentiation and the fusion of myocytes into myotubes[64,73,74]. GABARAPL1 on the

other hand is an autophagy gene, and the protein is present in many locations of the muscle cell[75]. GABARAPL1 has been associated with the formation and maturation of autophagosomes and it is likely that the increased expression of GABARAPL1 potentiates the fusion of myocytes, facilitating the formation of myotubes by its presence in the cytoplasm and its autophagic role[75,76]. This isolated capsaicin treatment did not induce any other changes in the expression of the genes investigated linked to cellular remodelling, BNIP3 and FBXO32.

When 100nM capsaicin was added to the medium in combination with dexamethasone both the protein content and the protein:DNA ratio were dramatically increased above the levels seen when dexamethasone was used in isolation. Of the four concentrations of capsaicin that were investigated, this concentration was the only one that reached a significant elevation above the protein content and protein:DNA ratio of the dexamethasone-only treatment. Changes in gene expression of the myotubes were therefore assess using 100nM capsaicin in combination with 50μM dexamethasone and compared to dexamethasone-only and untreated control. Interestingly, the addition of capsaicin to the dexamethasone does not induce significantly different results from what was seen using dexamethasone alone. However, while statistical significance were not reached capsaicin seem to somewhat counteract the dexamethasone-induced effects, bringing gene expression closer to the levels seen in the control cells. This is particularly clear with the expression of MYOD1, where dexamethasone-only is significantly elevated above the expression in control cells. The combined treatment on the other hand is not significantly higher than that of untreated cells, nor is it significantly lower than the dexamethasone-only treated cells. The expression of MYOG also shows this trend. While none of the treatments are significantly different

from one another, the expression seen with dexamethasone treatment is halved compared to the control levels, and then brought up to the same level as control with the combined treatment. The increased protein content, protein: DNA ratio and the normalisation of the gene expression in genes altered with dexamethasone-only treatment might be the result of stimulated protein synthesis through the activation of mTOR and its targets by the increase in intracellular calcium concentration through the TRPV1. These results thus indicate a potential for capsaicin to protect against dexamethasone-induced changes in gene expression and the resulting atrophy. However, this experiment needs to be repeated to confirm these findings as the data set is limited by a high sample variance which could account for some of the discrepancies seen in the dexamethasone data when comparing the gene expression between the first experiment in section 3.3 and to the second experiment in section 3.4. Being a pilot study there were also direct differences in methodology between these two experiments. The seeding density, treatment length and the time point at which the respective treatments were added to the cell culture differed slightly between them and this may well have caused some of the discrepancies seen here. This is the first study to our knowledge to assess the gene expression changes that take place in human skeletal myotubes when treated with dexamethasone, capsaicin or a combination of the two. As protein levels related to each gene were not measured and the Akt/mTOR pathway was not further investigated, the mechanisms behind this proposed protective effect by capsaicin on dexamethasone-induced atrophy remain unclear. The findings of the current study highlights a potential effect of capsaicin on muscle function and the response to dexamethasone-induced atrophy, and show the need for future work.

In addition to investigating the effects of dexamethasone and capsaicin this current study also sought to validate the atrophy-inducing effects of TNFα in this cell line as the role of TNFα and other pro-inflammatory cytokines in the skeletal muscle atrophy seen in relation to cancer have been greatly disputed[13,58,77,78]. Addition of 20ng/ml TNFα to the differentiation medium was shown to inhibit differentiation and myotube formation in human primary cells, and it has been shown to induce atrophy through the NF-$_K$B pathway and the activation of p38 MAP kinase[59,60]. The activation of both NF-$_K$B and p38 MAPK and their translocation to the nucleus results in the increased expression of both MuRF1 and FBXO32 as well and stimulates protein breakdown and decrease protein synthesis through the FOXO mediated atrogens[2,13,56,58–60,79,80]. In addition, TNFα treatment has also been proposed to induce skeletal muscle atrophy through the increased levels of ceramides[58,80]. Treatment with 15ng/ml TNFα was found to reduce surface area of C2C12 and L6 myotubes, as well as result in decreased protein synthesis, increased protein degradation by the up-regulated activation of FOXO and expression of FBXO32 but without inducing any change in MuRF1[58]. The 12-hour TNFα treatment, also lead to decreases in the protein contents of myosin heavy chain and eIF3-f. These TNFα-induced atrophy-responses were inhibited when ceramide-synthesis inhibitors were administered, indicating the role of ceramide synthesis in TNFα-induced atrophy. In the current study myotubes were treated with 10, 20 and 40 ng/ml TNFα and DNA content, protein content and protein: DNA ratio as well as gene expression were measured. Contrary to some of the previous studies these concentrations failed to induce atrophy in these human skeletal myotubes and resulted in no changes in protein content, protein: DNA ratio or the expression of TNNT1, MYOD1, BNIP3, GABARAPL1 and FBXO32. The 10ng/ml TNFα did however result in a significant decrease in total DNA content, but with no change to the protein:DNA

ratio. In agreement with the findings of De Larichaudy et al (2013) the myotubes did however express significantly lower levels of MYH2 when treated with 40ng/ml TNFα, compared to the untreated controls. This concentration is much higher than that used by De Larichaudy et al (2013) and while their results showed no increase in MuRF1 expression, it is possible that this TNFα treatment affected MuRF1 ligase activity but MuRF1 expression was not measured. Based on these results and the lack of atrophy seen TNFα treatment cannot be recommended as a method to induce atrophy in these human skeletal myotubes. It is however plausible that the results seen in this study is due to the use of the wrong concentrations, although concentrations of 15-20ng/ml TNFα have been used previously in both murine and human primary cell lines. The serum levels of TNFα in patients with gastric cancer and colorectal cancer have been shown to be within a much lower concentration, 3.7±7.3 pg/ml[57] and 22.3±13.9 pg/ml respectively[81]. Although the levels of circulating TNFα may not accurately reflect the levels of the cytokine within the muscle[49,79] the lower levels seen in cancer patients might be closer to the optimal atrophy-inducing concentration of TNFα. Thus optimization experiments to elucidate the adequate concentration of TNFα to induce atrophy in this cell line needs to be conducted and the processes involved further investigated.

Atrophy of skeletal muscle have been shown to result in the targeted destruction of mitochondria through macroautophagy, mitophagy,[13,19] and several rodent models of cancer cachexia have found an impairment of mitochondrial function and decrease in mitochondrial numbers, as well as decreased expression of peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PGC-1α) in the skeletal muscle of tumour-bearing mice[6,82]. Up-regulation of mitochondrial E3 ubiquitin protein ligase 1

(Mul1) has been shown to decrease mitochondrial function and to promote the fragmentation and destruction of these organelles during muscle atrophy induced with myostatin and with dexamethasone but also during denervation and starvation[19]. With the knockdown of Mul1 expression Lokireddy et al (2012) found that the mitochondrial fragmentation and degradation seen in control cells, as a result of these treatments with myostatin or dexamethasone, were inhibited in C2C12 myotubes and instead mitochondrial fusion was promoted. The study showed that Mul1 acts as an E3 ligase for the mitochondrial fusion protein Mfn2, which regulates mitochondrial fusion and morphology[83], and Mul1 was proposed as a key regulator of the mitophagy seen with atrophy[19]. As Mul1 is under the control of FOXO, like BNIP3, MuRF1 and FBXO32, the activation of this transcription factor seem to be the key regulator for many of the effects seen in skeletal muscle atrophy. The potential of capsaicin as a mediator of muscle wasting is even more intriguing when the disruption of mitochondrial function during atrophy is considered, as it was recently shown that the capsaicin-mediated activation of TRPV1 up-regulated PGC-1α in mice[84]. This research showed that TRPV1 was present in both mice and the C2C12 mouse cell lines, and could be activated by capsaicin. The elevation of $[Ca^{2+}]_i$ through capsaicin-induced activation of TRPV1 in C2C12 myotubes led to an increase in the expression of PGC-1α, enhanced mitochondrial biogenesis and ATP production. In mice this TRPV1 activation also induced up-regulation of PGC-1α expression, and in addition led to an increase in mitochondrial number and skeletal muscle remodelling. This remodelling was confirmed with the increased percentage of type I oxidative muscle fibres, the increased oxidative respiratory and endurance capacity seen in these mice compared to controls[84]. Taken together the study by Luo et al (2012) therefore provides another role for capsaicin-induced TRPV1 activation in skeletal muscle maintenance and function, but

also highlights the need for further study. As the current study successfully achieved the staining of mitochondria in the human myotubes using MitoTracker Green, measuring the effects of capsaicin and dexamethasone on mitochondrial content using this dye could be a next step to elucidate the effects of capsaicin on skeletal muscle function and its role in the prevention of dexamethasone-induced atrophy.

Menconi et al (2008) highlighted differences in the response to dexamethasone treatment in L6 and C2C12 myotubes, and Owens et al (2013), in addition, demonstrated differences in differentiation capacity and gene expression between four human skeletal muscle cell lines. Some of these differences can be attributed to the different mammal species, but as in the case of the study by Owens et al (2013) donor characteristics are likely to influence the characteristics of the cells. The profuse variability between people most likely play a role in cellular responses, with factors such as gender, age, ethnicity, health and lifestyle being able to influence results. The current study was conducted using one line of human skeletal muscle myotubes, i.e cells from one donor (n=1). Therefore to increase the reliability of these findings and to reduce some of the variability seen in these data, the study should be repeated using cells from more than one donor, taking care to minimise the variability in donor characteristics when doing so. As the current study was designed for pilot data collection and method development, there were some inconsistencies in the methodology used for the different experiments, with variations in seeding densities, treatment length and the time point at which the treatments were added to the differentiation medium. Thus future work should also try to limit these inconsistencies. One major limitation to the current study was that role of the TRPV1 was not investigated. The use of a TRPV1 antagonist, for example capsazepine, would greatly

increase the understanding the action of capsaicin and elucidate the mechanisms involved in the cellular response to capsaicin. In summary future work would include; repeating these experiments under standardised conditions, further investigation of the optimal concentration for TNFα to induce atrophy in these myotubes and to explore the effect of capsaicin in this setting, investigation of the effects of lower concentrations of capsaicin in dexamethasone-induced atrophy, and the effect of pre-treating of capsaicin before the induction of atrophy; as well as measuring protein levels and activation of the E3 ligases MuRF1, FBXO32 and further investigate the effects of capsaicin on protein synthesis.

Chapter 5: Conclusion

In conclusion the findings of this study support previous research and show that this line of skeletal muscle myotubes is a useful model for measuring dexamethasone-induced atrophy, and changes in gene expression, DNA, protein and protein:DNA ratio levels from the dexamethasone treatment. In addition, capsaicin influences the expression of myosin heavy chain and seems to moderate dexamethasone-induced atrophy in these human myotubes. TNF-α at the concentrations used in this study failed to induce atrophy in this cell line and had no overall effects on DNA and protein content, protein:DNA ratio nor did it induce any significant changes in the expression of the genes measured. The current study points toward a potential for capsaicin to normalise gene expression during dexamethasone-induced atrophy in this line of human myotubes, however to fully understand the mechanisms and processes involved, and its role on protein turnover further research is needed.

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