The roles of muscle contraction and pharmaceuticals on restoring glucose uptake in a mouse skeletal muscle model of immobilization

Euan Craig Kirkpatrick, BSc. (Hons),

MSc

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

(September 2023)

Abstract

Current understanding of muscle atrophy during immobilization and weightlessness is incomplete, partially due to the barriers to entry in the study of whole muscle. For this reason, a readily available model based on the C2C12 cell line is highly desirable as it would significantly increase the number of researchers able to engage in study of changes to skeletal muscle as a result of inactivity. The work presented here was in part funded as part of a newly established Astropharmacy cohort, which aims to tackle problems facing future manned space exploration. As such, the experiments performed here were planned and analysed with this perspective in mind. Inactivity is known to result in both muscle atrophy and insulin resistance. Terrestrially this is thought to be a key step in the development of diabetes as skeletal muscle is critical for the disposal of glucose during physical activity. For this reason, we are interested in establishing a pharmacological model of immobilisation with associated features including reduced glucose uptake (insulin resistance) and then restoring activity either by EPS (electric pulse stimulation) or further pharmaceutical intervention.

Chapter 2

Firstly, we confirmed the suitability of C2C12 cells in our hands as a platform for measurement of glucose uptake using 2-Deoxyglucose (2DG). We determined that at a final concentration of 25µM per well 2-deoxyglucose could be used to trace glucose uptake over a 24-hour period in both the basal and EPS treated state. We confirmed normal cell responses to EPS in terms of anabolic signalling changes, glucose uptake and lactate production. The ability of cells to respond to insulin was also tested and confirmed.

Chapter 3

After establishing the suitability of the platform, we were interested in determining the effect of repeated bouts of contraction throughout a 24-hour period effected the endpoints measured previously- glucose uptake, lactate output and anabolic signalling- as well as the addition of cell glycogen content. We found that there were no significant differences in glucose uptake, cell glycogen, lactate output or p-P70 (T389), p-4EBP1 (T37/46), p-mTOR (S2448) at the 24-hour timepoint with any of the frequency/ duration combinations that we attempted. This was attributed to a combination of the lack of a maintenance pulse during the rest periods and possible proximity effect.

Chapter 4

Returning to 24-hour continuous EPS we established the ability of combined CPA and blebbistatin (CB) treatment to inhibit contraction in c2c12 cells at concentrations of 100 µM and 10µM respectively. This was paired with increases in biomarkers (ATP2A1 and CALM1) that indicate elevated cytosolic calcium. When treated with EPS the CPA and blebbistatin model showed reduced glucose uptake, reduced P70, ERK1/2 phosphorylation and elevated 4EBP1 phosphorylation compared to cells treated with EPS alone, indicating prevention of contraction and consequent downstream effects in line with immobilisation.

Chapter 5

Lastly, we attempted to restore glucose uptake, glycogen, lactate output, anabolic signalling, p-EEF2 (T56), p-PKB (T3080), P-ERK1/2 (S217/221) to baseline values by application of combinations of EPS, AICAR and dantrolene. We found that application of these drugs reduced glucose uptake beyond what treatment with CB alone had. However, lactate output and markers of cytosolic calcium improved with dantrolene treatment. In cells that did not receive

CB treatment, we found that EPS had a neutral or negative effect in the case of AICAR on the ability of drug treatment to stimulate additional glucose uptake, which may suggest incompatibility between pharmacological and exercise-based uptake methods, possibly due to conflicting signalling between EPS which favours transient changes and drug treatments which favour sustained changes.

Acknowledgments

I would like to thank my supervision team for their guidance and expertise which I have benefited from during my studies at the university of Nottingham and has enabled me to produce this document. This is Dr Matthew Brook and Professor Paul Greenhaff from the School of Life Sciences, Professor Philip Atherton and Dr Daniel Wilkinson from the School of Medicine and Professor Philip Williams from the School of Pharmacy. I would also like to thank the technical staff throughout the university for making available the facilities and equipment required for this project. Additionally, I would like to thank the EPSRC for their part in funding the scholarship which allowed me to carry out the research reported here as part of the newly established Astropharmacy cohort.

Covid statement

During the period of March 2020 to September 2020 facilities at the university of Nottingham were unavailable due to the national lockdown measures imposed to combat the COVID 19 pandemic. As a result of this, training and practice of any laboratory techniques required for chapter 1 was not possible. Lack of access to mass spectrometer facilities at this time did not

have a direct impact on this work, however the queue for access caused by large numbers requiring access at once did affect this work.

Additionally, due to national and international shortages in DMEM, there was rationing of DMEM required in order to ensure equal availability of DMEM to departments, which necessitated slowing the rate of cell culture experiments until approximately December of 2020, which had an effect on the completion of chapter 1.

The above setbacks had a detrimental effect on the timeline originally envisioned for this work and so work that was planned for an additional chapter in which comparisons between the changes induced by the C2C12 model and human biopsies taken from volunteers in a bedrest study had to be scrapped.

Declaration

I declare that the data, analysis and discussion presented here is my own work unless stated otherwise. Works consulted in the creation of this document have been referenced appropriately. The data in this thesis was generated specifically for this Ph.D and has not been submitted elsewhere.

Table of Contents

Abbreviations

2DG- 2-Deoxyglucose

2DG6P- 2-Deoxyglucose-6-Phosphate

4EBP1- The binding factor of Eukaryotic translation initiation factor 4E

AA- Amino Acid

ADA- American Diabetes Association

AGE- Abnormal Glycation End Products

AKT/ PKB- Protein Kinase B

AMPK- Adenosine monophosphate activated kinase

ARED- Advanced Resistive Exercise Device

ATCC- American Type Culture Collection

ATP- Adenosine Triphosphate

C2C12 – mouse myoblast cell line

DHPR- Dihydropyridine Receptor

EE- Endurance Exercise

EPS- Electric Pulse Stimulation

ET- Endurance Training

FAD – Flavin Adenine Dinucleotide

FBS- Foetal Bovine Serum

FOXO – Forkhead box (gene)

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

GLUT # - Glucose transporter type #

HB- Homogenization Buffer

HBSS- Hank's Buffered Salt Solution

HIIT- High Intensity Interval Training

HK2/II- Hexokinase 2/ II

IGF-1 – Insulin like growth factor 1

IL-6 – Interleukin 6

INSR- Insulin receptor

IR- Insulin Resistance

ISS- International Space Station

LEO- Low Earth Orbit

MAFbx – Atrogin-1

MARES- Muscle Exercise Research Exercise System

MPB – Muscle Protein Breakdown

MPS – Muscle Protein Synthesis

mTORC- Mammalian Target of Rapamycin

MuRF1 – Muscle Ring Finger 1

NAD/ β-NAD- Nicotinamide Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NF-κB – Nuclear Factor Kappa B

P70S6K- Protein Kinase that acts on the S6 Ribosomal subunit.

PEMF – Pulsed Electromagnetic Field

PGC1-α - Peroxisome proliferator-activated receptor-γ coactivator 1-alpha

PVDF- Polyvinylidene Fluoride

RE- Resistance Exercise

ROS – Reactive Oxygen Species

RYR- Ryanodine Receptor

SC – Satellite Cells

SM- Simulated Microgravity

SOD1- Super-Oxide Dismutase

STAT3 - Signal Transducer and Activator of Transcription 3

STZ- Streptozotocin

T1D- Type 1 Diabetes

T2D- Type 2 Diabetes

TBC1D1- TBC1 Domain family member 1

TBC1D4- TBC1 Domain family member 4

TBST- Tris Buffered solution with Tween (20)

TCA – Tricarboxylic Acid Cycle

TGF-β – Transforming Growth Factor Beta

TNF-α – Tumour Necrosis Factor Alpha

TRX1 - Thioredoxin

Chapter 1: General Introduction

1.0 Skeletal muscle in health and disease

Skeletal muscle makes up a considerable fraction of total human body mass (Hoppeler and Flück, 2002) and plays a key role in maintaining whole body metabolic health (Mukund and Subramaniam, 2019), postural control and locomotion (Prince *et al*, 1997). Skeletal muscle is comprised of a complex structure that is key to its function, as well as many of the properties that make it important to whole body health. Principally, muscle is a contractile tissue attached to the skeleton by tendons, enabling the generation of movement. At the basic level, muscle is comprised of multinucleated cells that contain many myofibrils of sarcomeres arranged in a linear fashion to provide contractile ability by means of their overlapping myosin and tropomyosin chains (Frontera and Ochala, 2014). These myofibrils are bundled into muscle fibres, with each muscle consisting of many bundles of muscle fibres. This highly simplified model of skeletal muscle explains its mechanical importance in day-to-day life but does not encompass the range of important metabolic roles muscle fulfils. For instance, skeletal muscle acts an important fuel store and is crucial in whole body glucose disposal, roles that are critical in maintaining whole body health.

There are still many elements of skeletal muscles metabolic actions that remain unknown, but the act of contraction itself is known to be important in maintaining healthy functionality, both in terms of mechanical function and metabolic function (Mu *et al*, 2001). In healthy skeletal muscle, an electrical signal arrives through a nerve junction in the form of acetylcholine. This activates the receptor, which functions as an ion channel, allowing entrance of Na^+ and efflux of K^+, depolarizing the cell membrane (Fagurlund and Eriksson, 2009). Depolarization proceeds down the fibre until it reaches the T-tubule, a deep trench in the membrane that brings the otherwise remote sarcoplasm close to the cytoplasmic plasma membrane, and by extension the extracellular matrix (Al-Qusairi and Laporte, 2011). Changes in the charge state of elements

within the membrane belonging to the DHPR (Dihydropyridine Receptor) cause the receptor to change conformation, entering the open state. In skeletal muscle (unlike cardiac muscle), the DHPR is directly physically connected to the RyR (ryanodine receptor) and so opens in tandem with the DHPR, allowing Ca2+ to exit the sarcoplasm and enter the cytoplasm (Rios and Brum, 1987; Hayashi *et al*, 1997). Once released to the cytoplasm, the increase in Ca2+ displaces Troponin from actin filaments upon binding to the EF-hand groups present (Walsh, 1983). This clears the way for the "crawling" binding of myosin to the actin filaments of a muscle fibre, which shortens the length of the muscle due to the Sarcomere arrangement, producing contraction (Rassier, 2017). This contractile mechanism influences much of the downstream signalling that is associated with healthy skeletal muscle signalling, including Ca^{2+} dependant enzyme activity, reactive oxygen species (ROS) production (Henriquez-Olguin *et al*, 2019) and GLUT recruitment (Yaspelkis *et al*, 1997).

In addition to its role in locomotion, skeletal muscle plays a vital role in glucose metabolism, responsible for the majority of postprandial glucose uptake (the other organs being the liver and brain). The effectiveness of muscle glucose uptake is highly influenced by the level of muscle contractile activity, with periods of inactivity resulting in rapid onset of insulin sensitivity and impaired glucose uptake. Insulin resistance is best described as an inability to respond to insulin to the fullest extent. It is of particular importance in skeletal muscle, the liver and adipose tissue. Impaired ability to take up glucose results in accumulation in the bloodstream. Since metabolism of glucose requires entry to the cytoplasm and eventually the mitochondria, glucose cannot be effectively disposed of in the insulin resistant state (McCracken *et al*, 2018). Not only does inactivity have negative impacts on glucose metabolism, but also on skeletal muscle mass, which is controlled by the balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Sandri, 2013). Contraction and functional overload (i.e. exercise) is a powerful

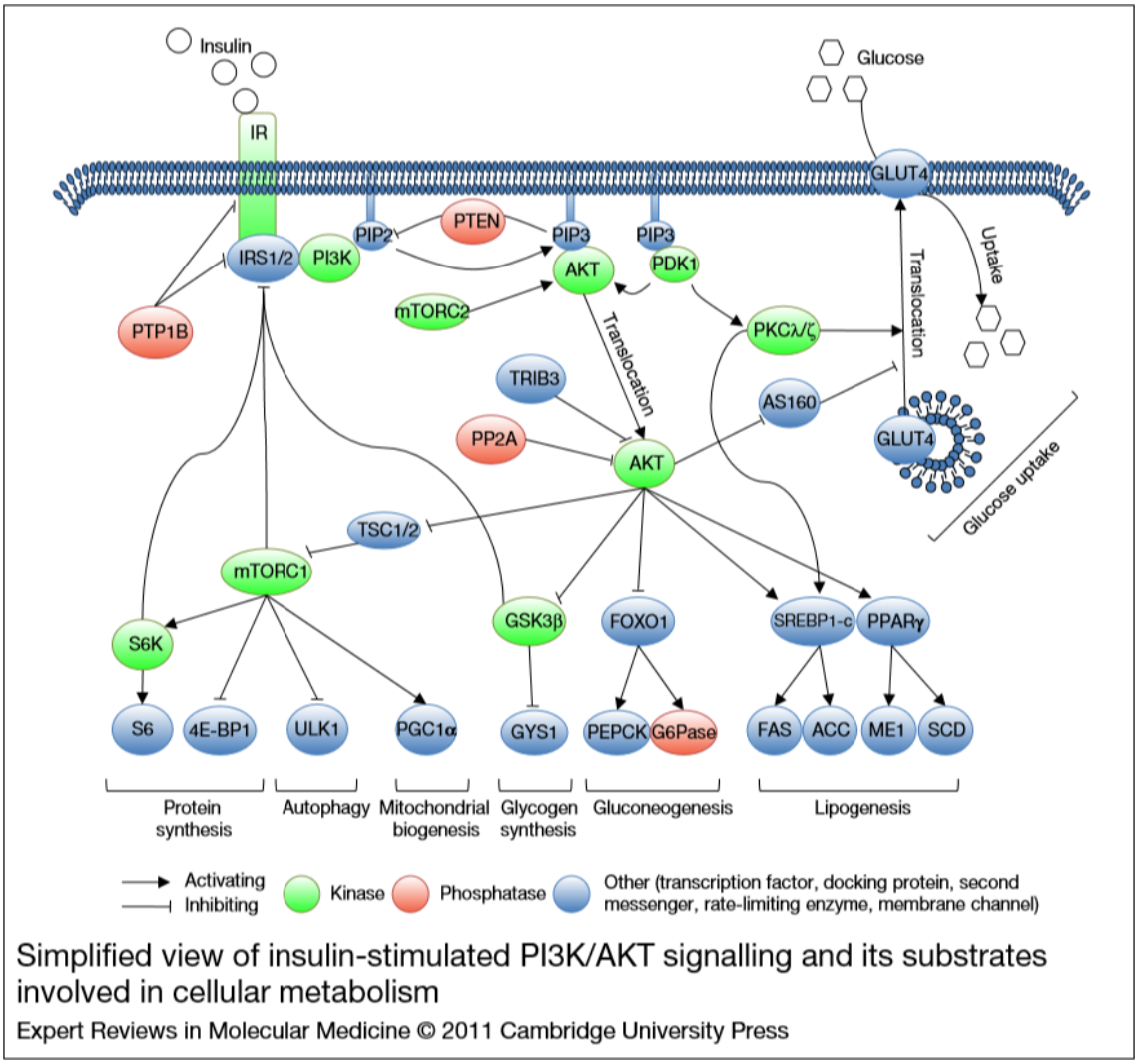
stimulator of MPS, whilst inactivity or immobilisation results in rapid suppression of MPS and muscle atrophy (Breen *et al,* 2013; de Boer *et al*, 2008; Shur *et al*, 2021). As such, not only does inactivity impair muscle glucose uptake, but reduces the capacity for muscle glucose disposal. Given the many important roles of skeletal muscle, it is therefore crucial to maintain muscle mass and metabolic functions throughout life. Periods of inactivity impact muscle insulin sensitivity, decrease muscle mass and strength, increasing the likelihood of falls (Granacher *et al*, 2013), reducing personal mobility and independence (Santos *et al*, 2016) thereby increasing overall all-cause death rate (Abramowitz *et al*, 2018; McPherron *et al*, 2013, Sasaki *et al*, 2007). Effective treatments against these effects of inactivity are of great importance, particularly with the frequent inactivity we experience throughout life due to disease, injury or sedentariness. The recent impact of lifestyle changes due to global pandemics has highlighted the potential significant reduction in population activity levels having large scale consequences (Narici *et al*, 2020). There are other challenges to muscle maintenance that most of the population will never experience. Astronauts undergo prolonged periods of inactivity during spaceflight and these challenges may be expected to increase with NASA's "design reference architecture" for sending life to mars indicating a manned mission of 31-34 months. As such, the requirement to treat inactivity is wide reaching. Our most effective strategies to combat these effects is often through exercise training regimes or pharmaceutical intervention, in which the negative outcomes associated with lack of contraction can be minimised or altogether eliminated.

1.0.1 Regulation of skeletal muscle mass

1.0.1 Akt mediated regulation of muscle mass

The primary determinant of skeletal muscle mass is the homeostatic balance between MPS and MBS. This balance is between pro-MPS factors such as the AKT/mTOR pathway, the stimulatory effect of dietary amino acid uptake, cytokine/myokine signalling and the pro-MPB effects of muscle disuse, ROS and inflammation (Rennie *et al*, 2003). Growth is regulated by several interconnected pathways with the purpose of balancing muscle synthesis rate against nutrient availability and requirement for physical activity. These pathways and constituent molecules can be classified as either catabolic (favouring muscle breakdown) or anabolic (favouring muscle synthesis) (McCarthy and Esser, 2010).

The main pathways credited with upregulating anabolic metabolism is the PI3K>Akt and mTORC1 pathways (Egerman and Glass, 2013; Watson and Barr, 2014; Bodine *et al*, 2001). The PI3K/akt pathway is particularly sensitive to hormones such as IGF-1 (Bahaskar and Hey, 2007) and insulin, muscle contraction (Sakamoto *et al*, 2003) -even weak activity such as stretching- as well as secreted cytokines and myokines (Carson, 2017). It is speculated that downstream of PI3K/Akt, calcineurin, a calcium sensitive phosphatase, is capable of initiating hypertrophy of skeletal muscle (Rusnak and Metz, 2000). This mechanism is responsive to extracellular signalling from prostaglandins and IGF-1, resulting in the stimulation of cell growth. The excitation- contraction coupling pathway (Wollheim and Sharp, 1981) is known to be of critical importance, but the exact mechanism is yet to be agreed upon, with some arguing that the beneficial effect of exercise (contraction) is by a separate – non Akt/PKB- pathway from the insulin response (Brozinick and Birnbaums, 1998; Mackenzie and Elliot, 2014). Others contend that there is an overlap at the Akt/PKB portion of the pathway, but that that effects of contraction are both dependant on time and fibre oxidation type and thus difficult to detect (Hu *et al*, 2018; Sakamoto



Simplified view of insulin-stimulated PI3K/AKT signalling and its substrates involved in cellular metabolism

Figure 1.1: Diagram of the downstream signalling of the Akt pathway in relation to insulin. The pro-anabolic and anti-catabolic processes of activation are matched with downstream outcomes. Diagram adapted from Schultze *et al*, 2012.

et al, 2002). What has also been established is that the mechanisms previously identified such as the Akt/PKB pathway, as well as the AMPK pathway (Mu *et al*, 2001; Vitzel *et al,* 2013) are not the only pathways that feed into glucose regulatory pathways and by extension, mediators of muscle synthesis and breakdown. The downstream effects of the activation of the Akt/PKB pathway combine pro-anabolic and anti-catabolic functions as shown in **figure 1.1**. mTORC1

represents a central regulator in cellular metabolism and can be activated by amino acids and contraction independently of AKT. The former group is represented by the action of mTORC1, which blocks the action of 4E-BP1, an inhibitor of transcriptional initiation (Musa *et al*, 2016) as well as initiating phosphorylation of S6, leading to increased MPS. The latter function is performed by AKt directly, which prevents the activation of the FOXO1 gene as well as downstream ubiquitin-ligases MAFbx and MuRF1b (Naehr and Bodine, 2014). Separate from the mTOR mediated pathways is the β-Catenin pathway, which is required for the growth of muscle in response to exercise (Armstrong and Esser, 2005). Maintaining muscle mass is energetically expensive, resulting in 30% of the heat output seen in cattle (Gregg and Milligan, 1982) and approximately $1/5^{th}$ of the basal metabolism of mammals (Hawkins, 1991). As a result, glucose uptake and insulin signalling play a role in maintenance of muscle mass, with insulin exerting strong anti-catabolic effects on its own (chow *et al*, 2006), as well as anabolic effects when present in conjunction with excess essential amino acids (Yoon, 2016). Phosphorylation of P70 by mTOR is necessary for hypertrophic response of muscle to progressive overload exercise. Hypertrophy is beneficial for the disposal of glucose both because of the greater sinking ability of greater masses of muscle and because of changes that occur in the mitochondria as part of the hypertrophic response (Merz and Thurmond, 2020). For this reason, the change in mTOR, 4EBP1 and P70 phosphorylation with EPS (Electric pulse stimulation) is of interest in understanding potential links to glucose uptake.

This does not mean that catabolic pathways are entirely undesirable, though overexpression of MAFbx and MuRF1b *can* lead to self-perpetuating atrophy thanks to associated downregulation of mTORc 1 (Folleta *et al*, 2011). Whilst muscle wasting is almost always associated with an increase in MAFbx and MuRF1b activity, it is not the case that increases in MAFbx and MuRF1b is indicative of atrophy (Baehr *et al*, 2014). A certain increase in (calcium controlled) proteolytic

activity is required for normal growth of muscle in response to exercise. This is thought to be required for remodelling the muscle so that it can function more efficiently at a cellular level (Cunha *et al*, 2012). The activity of ubiquitin ligases E1-3 can be classified as pro-catabolic. On the anti-anabolic side of muscle regulation there is AMPK pathway. The AMPK pathway exists to conserve energy in the event of starvation, as determined by the ratio of AMP (adenosine monophosphate): ATP (Adenosine Triphosphate) within the cell (Shaw 2009). As the level of AMP increases, AMPK increasingly downregulates the mTORc1 pathway. Inhibition of mTORc1 is achieved by phosphorylation of raptor, preventing complex 1 from forming (Kalendar *et al*, 2010). Additionally, TSC2 phosphorylation at multiple sites by AMPK inhibits mTOR1 complex formation and downstream anabolic signalling (Inoki *et al*, 2003; Sase *et al*, 2019; Leprivier and Rotblat, 2020). This has the effect of reducing protein synthesis rate, preventing mitochondrial biogenesis and increasing autophagy, all of which are hallmarks of deleterious skeletal muscle remodelling, or atrophy (Kelley *et al,* 2002; Sivitz and Yorek, 2010). Conversely, mTOR complex 2 is upregulated by activation of AMPK under low energy conditions. The result of this activation is increased PGC1-α (Peroxisome proliferator-activated receptor-gamma coactivator 1- alpha) activity (Cantó and Auwerx, 2013). PGC1-α is known to increase mitochondrial biogenesis as part of the cell survival promoting effects elicited by mTORC2 (Kazyken *et al*, 2019; Liang and Ward, 2006).

The key signalling changes that occur in response to muscle contraction that relate to muscle mass regulation are as follows. During the act of contraction pro-anabolic signalling is supressed in order to conserve ATP for contractile activity, resulting in suppression of mTOR by AMPK due to the energy stress of exercise (Deldique *et al*, 2005). In the post exercise phase, this is reversed, especially with excess amino acids, with increases in signalling though mTOR

(and subsequently P70 and 4EBP1) (Kumar *et al*, 2009), EEF2 (Dreyer *et al*, 2006) and ERK1/2 (Yu *et al*, 2001; Taylor *et al*, 2001).

1.0.2 Additional factors: Satellite cells, ROS and cytokine signalling

There are many contributing factors that can influence and feed into the pathways illustrated above. These factors have been suggested to have importance in muscle hypertrophy, potentially tipping the balance between MPS and MPB when it comes to the determination of whole- body muscle mass. Skeletal muscle is composed of postmitotic multinucleated myofibers and therefore doesn't undergo any cellular division. Instead, the addition of any new nuclei (termed myonuclei) arises from a pool of muscle resident stem cells called satellite cells (SC). Satellite cells spend most of their time in an Inactive "quiescent" state between the basal lamella and the plasma membrane of the associated myofiber (Hawke and Garry, 2001). When SC become activated, they undergo cellular division. One daughter cell becomes committed to the muscle fibre as a new myonuclei and the other undergoes self-renewal to maintain the SC pool for future use. Muscle SC have been shown to be essential for muscle repair in response to damage or injury, yet the role of SC in muscle hypertrophy or atrophy still remains a highly contentious issue. Decreased SC numbers and altered SC activation has shown to be common amongst conditions such as sarcopenic, unloading and cachexic muscle wasting, suggesting there is a possible intervention to be made with relation to SC function (McKenna and Fry, 2018). Many of the signal pathways that exist in myofibers also exist in SCs, such as IGF-1, glucocorticoid receptor, NFκB and TGF-β (Biressi and Gopinath, 2015) and they have the same effects as in their differentiated counterparts. However, whilst an individual myocyte is essentially replaceable, the death of a satellite cell which has not already produced a replacement leads to shrinking of the total pool of SCs in the body. The reduction in the SC pool

has been associated with a high degree of type II fibre atrophy that occurs with ageing (Verdijk *et al*, 2013; Day *et al*, 2010). Additionally, the presence of a SC reserve has been found to be required in mice for the long-term maintenance and repair of muscle after injury, immobilisation and exercise (Hinks *et al*, 2023), without this reserve the ability of muscle to undergo serial sarcomerogenesis is limited.

Oxidative stress, delivered in the form of ROS has been identified as another factor that Influences glucose uptake as well as the downstream mechanisms leading to muscle wasting (Winslow and Hall, 2019). However, there is subtlety in the interaction between reactive oxygen species and skeletal muscle that means no one blanket statement can always apply to the relationship between ROS and muscle wasting. A number of studies and reviews conclude that there were conditions under which ROS production could be associated with positive health outcomes, both locally and systemically (Thirupathi and Pinho, 2018; Biolo *et al*,2017; Radak,*et al*, 2016). Importantly, it is not the ROS that have a positive effect on overall health, but the endogenous antioxidant response that they induce. SOD1 (Superoxide dismutase) is one such antioxidant gene that is activated in response to exercise induced ROS. However, other genes such as TRX1 (Thioredoxin) (Manabe *et al*, 2014) are constitutively active in muscle, showing downregulation in response to immobilization in rats (Kawamoto *et al*, 2018).

Production of ROS is an unavoidable consequence of oxidative metabolism, which occurs in the mitochondria, of which there are many in skeletal muscle fibres (Kelley *et al*, 2002). Muscular ROS is primarily associated with NADPH in the sarcoplasmic space of sarcomeres. This has been seen to induce endogenous antioxidant activity, which provide health benefits not replicated by substitution with orally administered antioxidants Vitamin E and C (Ristow *et al*, 2009). These studies also conclude that it is the transient nature of the extra ROS production that limit the harmful effects when the source is non- exhaustive exercise (Duca *et al*, 2006). There is

compelling evidence to indicate that mitochondrial ROS is a signal to induce apoptosis and proteolysis (Hyatt *et al*, 2019; Ji and Yeo, 2019; Kumar *et al*, 2019) through a number of pathways including FOXO induced ligases (Li *et al*, 2003), Caspase 8 (Campbell and Quadrilatero, 2016) and likely the Ikk/NF-κB pathway (Israel, 2010). Recent studies have determined that during bedrest, mitochondrial ROS (H_2O_2) is not the cause of muscle wasting (Dirks *et al*, 2020; Marchant *et al*, 2020).

Defence against ROS produced in skeletal muscle is essential to maintain normal function throughout life, as shown by studies involving "antioxidant genes" SOD1 (Dobrowolny *et al*, 2008) and PRDX2 (Peroxiredoxin 2) (Olthoff *et al*, 2018), in which loss of antioxidant genes resulted in mice with atrophied muscle phenotype and reduced force generation respectively. Furthermore, both PRDX2 and TRX1 are implicated in normal response to glucose via the insulin responsive pathway, most likely protecting against oxidative stress and subsequent apoptosis signalling (Kim *et al*, 2018; Venojärvi *et al*, 2014). Knockout of the SOD1 gene resulted in β-cells with reduced performance, which in turn resulted in worse glucose tolerance than in wild type mice (Muscoguiri *et al*, 2013).

IL-6 is a cytokine which increases in muscle by two orders of magnitude during exercise. It Is also known to inhibit the AMPK pathway. The action of IL-6, among other, pro-inflammatory cytokines is thought to have a role in the development of diabetes, though it may simply be acting in its anti- inflammatory capacity against other cytokines such as TNF-α that are present in diabetes (Kristiansen and Mandrup-Poulsen, 2005). Increased IL-6 activates STAT3. High STAT3 activity induces FOXO and myostatin activity, as seen in studies centred on atrophy and cancer related cachexia. At least one of the final executors of this pathway is known to be the ubiquitin protease degradation system, the same system that is responsible for degrading STAT3, ensuring effects are only temporary (Radigan *et al*, 2019). IL-6 also has an important function in regulating

glucose release from the liver as well as the proliferation state of hepatocytes (Schmidt-Arras and Rose-John, 2016)

1.0.3 Distinctions between mechanisms of atrophy

Muscle atrophy occurs when the muscle protein synthesis rate does not equal or exceed the rate of breakdown. This can occur due to depression in the rate of synthesis or elevation in the rate of breakdown, though often both scenarios will be true.

Age related muscle loss (sarcopenia) is highly prevalent amongst older populations. The mechanism behind sarcopenic loss of strength include infiltration of lean mass by fat (Marcus *et al*, 2010), changes in mitochondrial energetics and fusion (Kim *et al*, 2017) and reduction in the overall rate of protein synthesis in a muscle group dependent manner (Miller *et al*, 2019). To an extent these mechanisms appear to be preventable through exercise training (Brightwell *et al*, 2019), however, denervation leading to permanent loss of granular motor control is not responsive to this type of intervention (Park, 2015), which is crucial in differentiating sarcopenic muscle wasting from disuse atrophy.

This differs from cachexic muscle wasting, which is muscle atrophy that is a secondary outcome of cancer or other disease, characterised by loss of mass and functional strength even in the face of nutritional supplementation (Fearon *et al*, 2011). Cachexic muscle wasting comes with a decrease in strength that is greater than can be accounted for by reduction in CSA alone and so it is derived that the "quality" of remaining muscle is reduced (Fanzani *et al*, 2012). This condition is difficult to distinguish from immobilization/ disuse atrophy by pathology alone because cancer patients will likely experience both, but the changes in MPS and MPB are

distinct (Evans, 2010). At least a part of this type of muscle wasting can be explained by reduced mitochondrial functionality induced by radiation therapy used in many cancer treatments (Pedrosa *et al*, 2023). This is distinct from muscle wasting caused by the cancer itself which are caused by secreted factors which cause deleterious activation of p53 (Ruan *et al*, 2023) and FoXO3 (Zhang *et al*, 2023) in muscle and mitochondria. This compounds with immobilization type atrophy, resulting in poor oxidative capacity. Furthermore, the irradiative treatment impairs the regenerative and regulative capacity of satellite cells (Amorim *et al*, 2020). Reduced functionality of SC is known to occur in chronic kidney disease related atrophy (Zhang *et al*, 2010).

Immobilization or disuse atrophy is caused by muscle unloading. It is prevalent among short- and long-term hospitalized cohorts as well as in astronauts. In both cases, mechanical unloading initiates changes in metabolism leading to adaptations which reduce functional ability (Brooks and Myburgh, 2014). Presumably the evolutionary basis for this adaptation is to reduce energy expenditure from tissue that is surplus to the individual's immediate requirement. Some propose that in space this adaptation would be self-limiting, bottoming out when "full" adaptation has been achieved (Stein, 2013). With consideration to skeletal muscle, there is evidence that this is likely true, however the reversibility of unchecked long-term adaptation is unknown, other tissues like the eyes and bone may be permanently altered (Lee *et al,* 2016; Sibonga, 2013) and it would be ethically difficult to allow adaptation of that magnitude to occur as it may preclude returning to gravity. This form of atrophy represents a major obstacle to the future of long-term space exploration, necessitating new countermeasures to improve muscle retention in space.

Mitochondrial changes, including reduction in the mitochondrial volume, lowered respiratory capacity and increased ROS production (Hyatt *et al*, 2019) occur as a result of muscle disuse for

any reason (sedentary lifestyle, immobilization, microgravity). Muscle contractile force is also compromised, owing to reduction in cellular actin, among other elements (Fitts *et al*, 2001). There is some evidence that unloading induced changes may arise from microRNAs that are expressed by immune cells in response to unloading (Teodori *et al*, 2018). Additionally, glucose uptake is compromised during immobilisation (Burns *et al*, 2021), and this persists for some time afterwards (Vissing *et al*,1988), with 21 days of bedrest in humans requiring at least two weeks of normal lifestyle to return to near normal insulin sensitivity and blood glucose (Heer *et al*, 2014). A possible point of overlap between changes in protein synthesis and glucose uptake is the actin and myosin structures required for GLUT4 vesicle trafficking and fusion to the membrane that occurs during insulin and contraction-based glucose uptake (Sartori *et al*, 2021; Bose *et al*, 2001). Because these elements are susceptible to regulation by similar mechanisms, inactivity could influence both. It has been shown in mice that preventing actin severing by genetic knockout of gelsolin results in changes associated with metabolic syndrome including increased adiposity and reduced glucose tolerance (Gertz *et al*, 2018).

1.1 Regulation of skeletal muscle glucose metabolism

1.1.1 The fate of consumed glucose

Glucose is an essential fuel used primarily by the brain, liver and muscles. In skeletal muscle, glucose is used as a substrate for the production of ATP to facilitate locomotion and posture control. For this reason, the ability of skeletal muscle cells to take up glucose from the surrounding blood vessels is of paramount importance. Dietary glucose is absorbed through the small intestine by transport through the gut wall by STGL and GLUT 2, with assistance from a number of cation transporters maintaining membrane potential to modulate glucose

transport rate (Chen *et al*, 2016). From this point, glucose makes its way from the extracellular matrix (ECM), through capillary networks to general circulation. Factors such as the time of

consumption, exercise status, meal carbohydrate content and transport

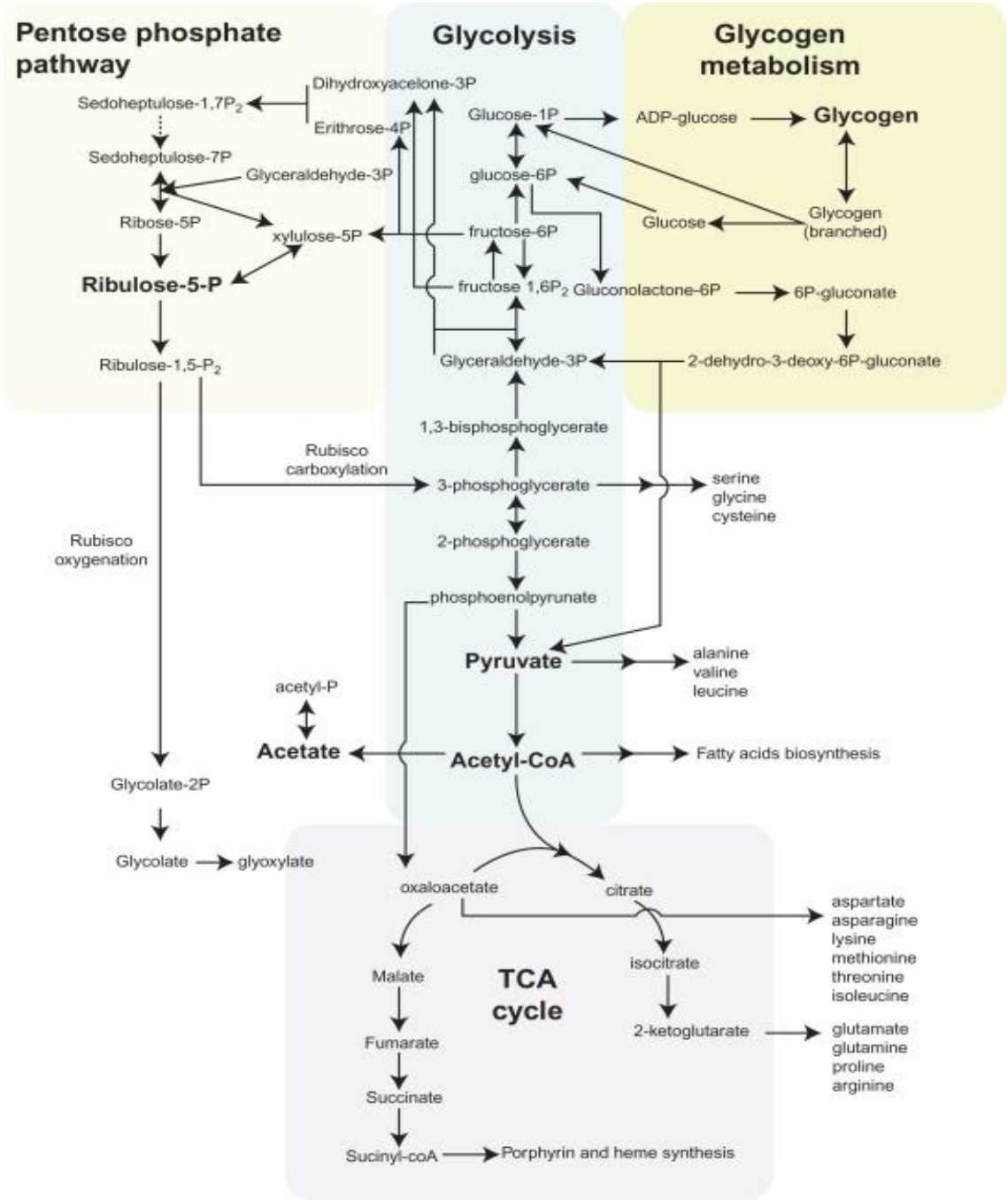


Figure 1.2: Diagram of the intermediate stages of glycolysis leading into the TCA cycle, showing overlap with glycogen storage. Also shown are some of the opportunities for TCA cycle intermediates to be used in the synthesis of amino acids. Figure adapted from Litwack, 2018.

rate determine the size of the resulting blood glucose excursion (Franc *et al*, 2010).

Under low energy conditions, such as starvation, or prolonged moderate intensity aerobic exercise, muscle cell glycogen stores are depleted (Knuiman *et al, 2015*). In response to this, glucose transport into skeletal muscle is increased (section **1.2.3**)**.** Once inside muscle cells, glucose can be directly utilized as fuel for ATP generation, or it can be used to (re)synthesise glycogen stores within the muscle. The "global" glycogen store of a myofiber can be broken down into three compartments or "pools". These are the Intramyofibrillar, intermyofibrillar and subsarcolemmal stores (Gejl *et al*, 2013). The muscular glycogen store is largely independent of post-prandial glucose, which is handled by the liver (Adeva-Andany *et al*, 2016). Unlike in skeletal muscle, liver glycogen can be released back to the bloodstream for use by other tissues by the process of glycogenolysis (Jensen *et al,* 2011).

Glucose that is immediately required as a fuel, either after transport across the membrane or having been liberated from glycogen stores undergoes glycolysis, which converts it to pyruvate so that it can be used in the TCA (tricarboxylic acid) cycle. Glycolysis is initiated by the phosphorylation of glucose by hexokinase II. This confines the molecule to the inside of the cell (Fueger, 2005). The sequential action of phosphoglucose isomerase, followed by phosphofructokinase results in production of fructose-1,6- bisphosphate. By this point 2 molecules of ATP have been consumed to facilitate phosphorylation and conversion (Melkonian and Schury, 2019). Fructose-1,6- bisphosphate is cleaved by aldolase into two distinct sugars. Triosephosphate acts on one of these, converting it to glyceraldehyde-3-phosphate, the same as the second product of cleavage. Utilizing NAD^+, both molecules of glycerldehyde-3-phosphate are phosphorylated to 1,3-bisphosphoglycerate. Phosphoglycerate kinase converts these 2

molecules of 1,3-bisphosphoglycerate to 2 molecules of 3-phosphoglycerate, producing 2 molecules of ATP in the process. 3-phosphoglycerate is isomerised to 2-phosphyglycertae, which is then dehydrated by enolase, giving 2 molecules of phosphoenolpyruvate. Pyruvate kinase strips the phosphate from phosphoenolpyruvate, producing 2 molecules of pyruvate and 2 molecules of ATP, for a total of 4 glycolytically produced ATP. This process is depicted in **figure 1.2.**

The TCA cycle, which utilizes pyruvate produced by glycolysis requires oxygen to fully convert pyruvate to CO_2 by way of Acetyl CoA (Adeva-Andany *et al*, 2016). In the event that there is insufficient oxygen supply to regenerate NAD^+ and FAD, pyruvate will instead be converted to lactic acid (Berg *et al,* 2002). The degree to which a muscle fibre can maintain oxidative metabolism during exercise – by maintaining the Acetyl CoA branch of the pathway- is a key characteristic differentiating type I and II fibres (Ivy *et al*, 1980). The purpose of the TCA cycle is twofold; it produced ATP energy molecules to fuel other activities within the cell, whilst also generating metabolites that can be used by other pathways such as amino acid synthesis. By the action of a number of isomerases and kinases, Pyruvate is converted to citrate, followed by cis-aconitrate then isocitrate. This removes two molecules of water per molecule of pyruvate fed into the cycle. Isocitrate is then oxidised, forming the intermediate product Oxalocuccinate, which is then decarboxylated, forming α-ketoglutarate. Both oxidative and decarboxylating functions are performed by isocitrate dehydrogenase. This step is notable for being the bottleneck in the cycle, as well as an irreversible reaction. A molecule of CO_2 $NADH^+$ and a single proton is produced (Al Khallaf, 2017). α-ketoglutarate is converted to succinate by way of succinyl-CoA, producing another molecule of NADH with a proton, a molecule of ATP and CO_2. From here, Succinate is built back up to oxaloacetate with fumarate and malate as conversion

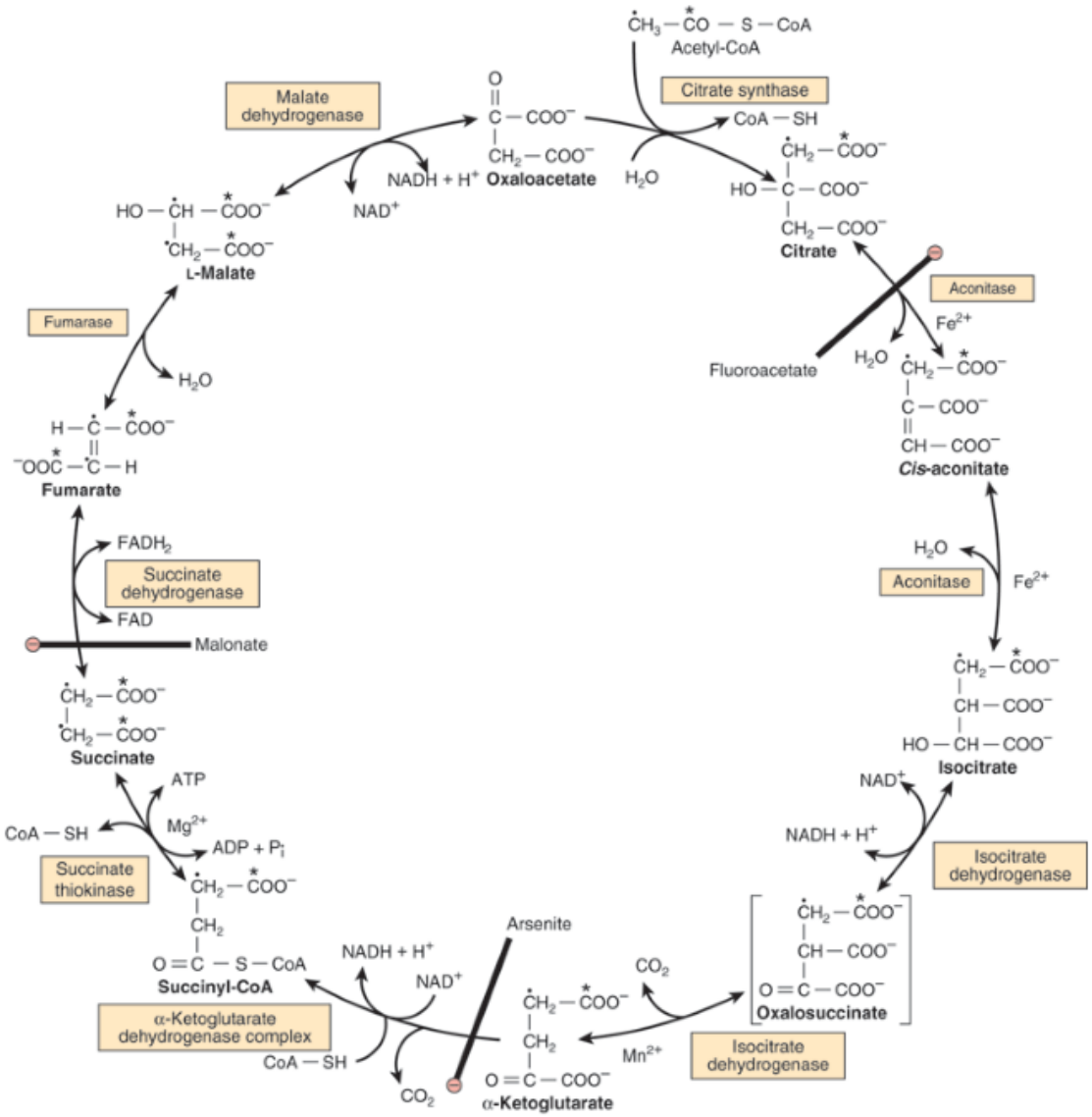


Figure 1.3: A detailed depiction of the TCA cycle, including the primary respiratory products, CO_2, H_2O, ATP and $FADH_2$. Not shown are the points at which amino acids can feed into the cycle or be produced from the cycle's components. Image adapted from Murray *et al*, 2009.

steps. This cycle is shown in detail in **figure 1.3**. Importantly, most of the conversion steps of the cycle are reversible, allowing a great degree of flexibility in order to provide carbon skeletons required to maintain the cycle, despite the lack of a central storage organ for amino acids

(Wagenmakers, 1998). This flexibility also allows for siphoning of carbon skeletons for glutamine synthesis, required for wound healing, immune function and fat metabolism in adults (Watford, 2015). Dysregulation of the TCA cycle and associated amino acid metabolism is associated with neuropathy in those with diabetes, with type 2 being affected more so than type 1 (Mathew *et al*, 2019). Blood concentration of amino acids have been found repeatedly to be a good indicator onset of diabetes (Welsh *et al*, 2018) and of clinical outcomes (Gar *et al*, 2018; Oresic *et al*, 2008), despite being clinically unsuitable at the moment due to being dependent on an individual's transient glycaemic status.

1.2 The importance of Calcium

Calcium is an important regulator of activities within cells, but especially in excitable cells like skeletal muscle, as outlined above in which the passage of the Ca^{2+} from the sarcoplasmic reticulum through the ryanodine receptor (RyR) initiates actin-myosin bridge crawling. However, the cytosolic calcium concentration of the cell also effects the activity of many other proteases and kinases. Many of these are required for the normal development and maintenance of skeletal muscle, such as MEF2 and NFAT (Tu *et al*, 2016). During normal physical activity, the calcium concentration within the cytoplasm rapidly elevates and then returns to normal in what is called a calcium transient. The return of calcium to the sarcoplasmic reticulum is achieved by the SERCA2 ion channel pump. The tight regulation of cytoplasmic calcium enables rapid transients which allow for the recruitment of muscle to perform physical activity without modifying the calcium concentration in the long term.

However, there is increasing evidence that shows that lingering changes in calcium handling elements of skeletal muscle play a role in changes that occur within muscle, both beneficial adaptations and deleterious pathological changes. For example, after strenuous exercise there

is a low-level leak in calcium caused by reduced FKBP12 – a stabilising unit withing RyR1- which confers a beneficial resistance to fatigue in muscle. After the adaptation period (approximately 6 weeks in mice) the leak disappears, but fatigue resistance remained, attributed to an increase in SERCA content (Sedwick, 2019). This is in opposition to other findings which show that in humans with malignant hyperthermia- a disease characterised by calcium leak due to RyR mutation resulting in chronically elevated calcium- there is a tendency to develop hyperglycaemia and diabetes (Nelson *et al*, 2020).

Clearly, there is a space within our understanding of the regulation of calcium in the normal function of skeletal muscle that has yet to be fully explored, most likely because when it is functioning normally it is unnoticeable. Understanding the boundary at which acute and chronic calcium derived changes occur and if these are dependent on other mechanisms would be of great interest to the prevention of diabetes on earth and in maintaining the health of astronauts and other immobile cohorts.

1.3 Disuse and Muscle health

Muscle disuse has major implications for human health, both terrestrially and in a microgravity environment, however the major difference between environments is in which branch of the pathology is more dangerous. Terrestrially, muscle disuse is at its most severe in long term or permanently bed bound/ immobilized individuals, however the requirement to support the body against gravity is still present, even if it is to a reduced extent. This means that on earth the main threat posed to health by disuse is at the population level in the form of the development of insulin resistance and subsequently type 2 diabetes (Taylor, 2012) except possibly in those with advanced sarcopenia. The disease progression not only reduces quality

of life for the individual, but places massive strain on publicly funded health systems. This is in contrast to the microgravity or "outer space" environment in which the physical loss of capability is most dangerous, rather than the risk of diabetes. This is because unlike on Earth, there are no large scale social safety nets to provide assistance to people with reduced physical ability, meaning that it is critical that astronauts must always be capable of strenuous activities such as setting up shelters and Extra vehicular activity (EVA) in which each action requires overcoming the resistance of a space suit that is kept stiff by breathable air that fills the suit (Rai *et al*, 2012). A manned Mars mission has the requirement to function at full capacity after what amounts to up to 34 months of muscle disuse, with no prospect of assistance or removal from the situation, meaning current understanding of disuse atrophy is likely insufficient to design an effective, sustainable countermeasure. To determine the requirements of the countermeasure we must first understand the effects of disuse on both contractile and metabolic ability of muscle.

1.3.1 Disuse Models and Muscle size and function

Human models of spaceflight rely on either voluntary bedrest, dry immersion, or immobilization to reproduce the unloading conditions experienced in orbit (Jost, 2008). Of these three methods, head down bedrest and dry immersion most accurately reproduces whole-body effects, such as fluid redistribution towards the head (Trappe *et al,* 2006; Tomilovskaya *et al*, 2019). Both the Torq and strength of the knee are known to decrease by approximately 5% per week of bed rest (English and Paddon-Jones, 2010). This is accompanied by a reduction in muscle volume of around 10% in both the gastrocnemius and soleus (Akima *et al*, 1997). Increases in muscle protein breakdown related factors like MuRF1 and Atrogin 1 are only seen briefly and in the early phase of bedrest, leading to the conclusion that reduction in synthesis is the most important factor in determining muscle mass and function loss during

disuse (Brocca *et al*, 2012). Additionally, bedrest disuse results in elevated insulin, which is thought to maintain mTOR activity at normal levels despite the reduction in synthesis of contractile elements. The drawback of this method is that it is time and resource intensive, requiring supervision and extensive support for participants as maintaining a reclined posture for extended periods is mentally and physically taxing (Berg *et al*, 1991). Immobilization is cheaper and less taxing for the participants but does not reproduce whole-body effects. Animal models also exist in the form of the hindlimb suspension model, which uses rats. In this model, microgravity conditions are simulated only in the rear half of the rat, which is suspended by the tail via attachment to a ceiling mounted armature that permits the rat to move freely using its forelimbs (Chowdhury *et al*, 2013). Both human and animal models succeed in reproducing the muscle atrophy and bone density loss observed during spaceflight. The "diabetogenic" aspect of spaceflight is also reproduced using *in vivo* models (Tobin *et al*, 2002; Dirks *et al,* 2016; Kwon *et al*, 2016).

1.3.2 Disuse Models and Muscle Glucose Uptake

As well as reducing the mass and strength of muscle, disuse also reduces the quality of the muscle as well. In bed rest studies it has been seen that metabolic inflexibility, the inability to change substrate oxidation between glucose and lipids (and the reverse) sets in after only a few days, though this appears to be partly dependant on energy balance (Rudwill *et al*, 2018). Inactivity is associated with reduction in ability to store glycogen, oxidise glucose (Mikines *et al*, 1991) and recruit GLUT4 and HKII (Biensø *et al*, 2012). Though the formation of insulin resistance and metabolic inflexibility during bedrest was initially thought to be caused by abnormal deposition of intramyocellular lipids (IMCL), it now seems that this is not the case and that pyruvate dehydrogenase 4 is a key player alongside alteration in normal calcium handling, namely excessive prolonged leak from the sarcoplasmic reticulum into the

cytoplasmic space (Shur *et al*, 2022). There is still some disagreement as to the extent to which the muscle fibre type shift from type I to type II is the cause of reduced oxidative capacity or a symptom of that reduced capacity (Plotkin *et al,* 2021).

Importantly, the reduction in metabolic (insulin stimulated) ability as well as the aforementioned contractile ability do not occur in isolation. Both of these changes are subject to regulation by the calcium concentration within the cytoplasm. Additionally, physical activity reinforces the function of both contractile elements by signalling for remodelling as well as the metabolic elements by upregulation of oxidative elements such as PGC-1α and PPARγ which are beneficial in both the glucose uptake and lipid oxidation metabolic pathways. Furthermore, because of the overlapping nature of the contractile and insulin stimulated pathways around the cytoskeletal structures that are responsible for the alignment and fusion of GLUT4 bearing vesicles with the plasma membrane, remodelling that facilitates increased efficiency in either of these pathways should have a beneficial effect on the other.

1.4 Exercise for the prevention of deconditioning

1.4.1 Exercise for maintenance of muscle mass and strength

Current exercise interventions have achieved much, but the drawback is that a number of hours each day must still be assigned to maintenance of muscle mass rather than performing other duties. Initial exercise regimes were able to reduce muscle mass loss by approximately 50%, whilst newer methods have reduced the number of muscle groups that atrophy as well as minimised loss of muscle volume to at most a loss of 9.7% in 17-week bedrest studies (Shackelford *et al*, 2004). A number of papers discuss the overlap in conditioning caused by both resistance training and endurance training, with the aim of reducing training regimes to just resistance training (Steele *et al*, 2019), on the basis that it would conserve the limited

resources aboard the ISS -oxygen and electricity- whilst still maintaining astronaut fitness. This is an emerging concept in multiple exercise-treatment fields, including cancer survivor care (Sheehan *et al*, 2019) and cardiovascular disease treatment (Mcleod *et al,* 2019). The theory behind resistance exercise therapy in diabetes patients is that by maintaining muscle mass, whilst decreasing fat mass, the ability to dispose of glucose relative to body mass should increase, unlike unmanaged weight loss, in which both muscle and fat is lost (Kehsel and Coker, 2015).

1.4.2 Exercise for maintenance of glucose uptake

Skeletal muscle glucose uptake during exercise is an area of ongoing work, that has shown greater depth than perhaps expected. Chief among these is the fact that not all muscle contractions are equal. This may be partially linked to the unique characteristics shown by the different types of muscle fibre expressed in humans (Gaster *et al*, 2000). GLUT4 is the primary transporter recruited in response to aerobic exercise or endurance training (ET). Recruitment of GLUT 4 in response to ET is a well-established outcome (Tjønna *et al*, 2008; Shahgolian *et al*, 2015). The pathway for recruitment responds to calcium release, NO and AMPK activation (Richter and Hargreaves, 2013), with the end result being that GLUT4 storage vesicles (GSVs) translocate to and fuse with the t-tubule and sarcolemma membranes (Lauritzen, 2013) **(Figure 1.4)**. However, Evans *et al* (2019) contests that the same is not true of resistance training (RT), which favour and rely on type 2 fibres more so than type 1. They present a number of papers in which it was demonstrated that transport increased, but the presence of GLUT4 at the membrane did not. This does not preclude GLUT4 from playing a role in RT induced glucose uptake, as it is uncertain if the activity of basal GLUT4 transporters can increase or not

(Antonescu *et al*, 2005). The benefit of exercise dependent GLUT4 recruitment is that it is not impaired by insulin resistance, so an increase above the basal rate of glucose transport achieved by what might be called constitutive GLUT1 and GLUT4 is possible even in highly insulin resistant individuals (Goodyear *et al*, 1998).

Beyond the action of transporters, contraction induces changes in the secretion of myokines, which act to enhance mitochondrial biogenesis- and therefore oxidative capacity- as well as enhancing other tissues ability to respond to insulin (Lee and Jun, 2019). One of the long-term benefits of exercise training is the vascular remodelling that occurs to facilitate capillary formation. This adaptation improves glucose supply to muscle and takes longer than other factors to become reversed by muscle disuse (Laughlin and Roseguini, 2009). Additional pathways that allow for glucose uptake in response to exercise have been identified *i.e.* pathways that are not reliant on the PI3K/Akt or insulin pathway. The inclusion of the AMPK pathway -identified earlier (section 1.1.1) as being anti- anabolic- may seem counterintuitive, but it has been identified alongside ROS mediated pathways (Chambers *et al*, 2009) as stimulating GLUT4 recruitment during exercise. This can be rationalised as part of its energy regulating function, in which increasing fuel intake and fuel efficiency is attempted alongside minimising fuel use (figure1.4.)

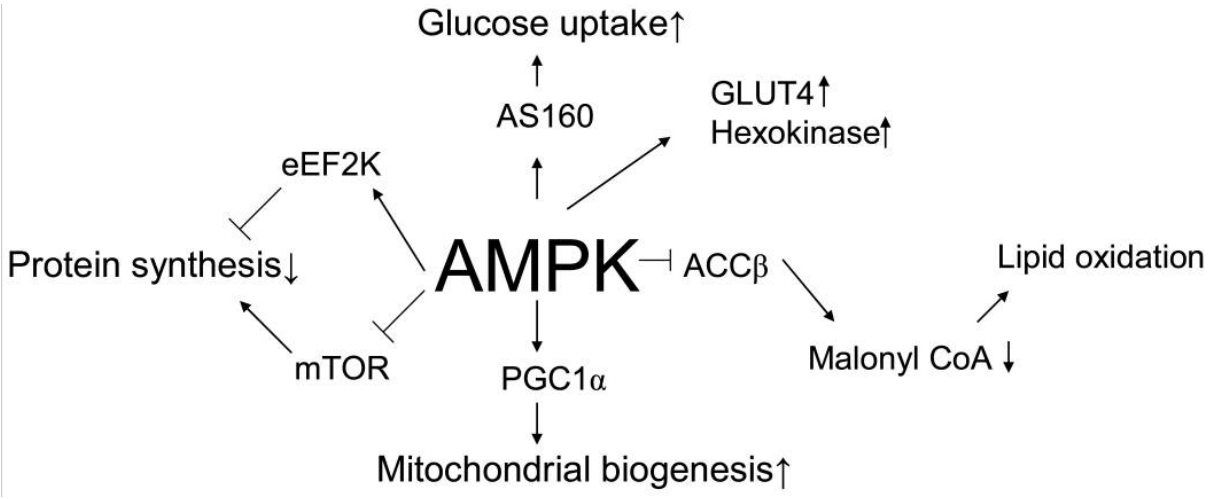


Figure 1.4: The theorized anti-anabolic and pro-biogenesis branches of the AMPK pathway derived from mice with daily pharmacological AMPK activation. With controlled nutrition, the mice showed phenotype similar to that of endurance trained individuals. Diagram adapted from Richter and Ruderman, 2010.

One potential danger on over reliance on the ability of exercise to stimulate glucose uptake is that damage to muscle by or during eccentric contraction can prevent the uptake of glucose (Andersen *et al*, 2018). Under damaged conditions, glucose uptake though the insulin sensitive pathway is impaired. Though this arm is already impaired in disuse, in an environment such as space where the individual cannot leave the IR inducing environment, damage to muscle could pose a more serious risk than on earth.

1.4.3 Potential problems with exercise as a disuse intervention

The main difficulty with researching the efficacy of exercise as a disuse countermeasure is the difficulty in accessing true microgravity. In the absence of true microgravity, there are

acceptable substitutes for human volunteer studies such as bedrest and dry immersion, but these are still resource intensive and so the number of institutes that can realistically expect to run a study like this are limited. Another issue with exercise as a disuse atrophy treatment is the variable response between individuals to exercise. A combination of the perceived difficulty of the exercise, feeding state, age and genetics of the participants can significantly alter the response to the exercise (Meyler *et al*, 2021). Additionally, it can be challenging to recruit participants for disuse atrophy studies due to the toll that immobilization takes on the body.

Exercise as a countermeasure to microgravity has seen some success, however it is difficult to optimise exercise parameters in real microgravity as the astronauts are there to perform other experiments and so elements such as hours spent sleeping, calorie intake and environmental stressors cannot be fully controlled, making it difficult to disentangle the effects of an intervention from other sources of variability.

1.5 Pharmaceuticals for the prevention of deconditioning

1.5.1 Pharmaceuticals for the maintenance of muscle mass

Pharmaceutical intervention to treat muscle wasting in space and on Earth is an attractive prospect for several reasons. As a matter of convenience, hours not spent maintaining body mass can be invested in other areas, be it work or health related. Secondly, pharmaceutical intervention, unlike exercise does not have the same required level of physical ableness that might not be present in someone facing muscle wasting, due to either immobilizing injury or other conditions (severe space adaptation syndrome or cancer cachexia). Due to altered pharmacokinetic and pharmacodynamic properties of drugs in reduced gravity (Eyal and Derendorf *et al,* 2019), the use of drugs in space should not be taken lightly, as unexpected

changes in potency and metabolism could form a potentially lethal combination in microgravity environments, which are isolated from external assistance. This is not to say that astronauts make little use of drugs whilst in space, in fact they are often used, especially during the initial adaptation period in which individuals will have trouble with blocked sinuses due to fluid re-distribution, sleeping and back pain (Kast *et al,* 2017). Of 24 astronauts included in a pharmaceutical use study (Wotring, 2015) 17 reported use of medication to assist sleeping and over half the population used analgesics for headaches.

In terms of pharmaceutical intervention for prevention of muscle mass loss on Earth there has been a lot of research, but few sustainable drug therapies. On Earth the main types of muscle loss that lead to clinical intervention are sarcopenia- age related- and cachexia- disease related. In the case of Sarcopenia, individuals lose muscle mass and fine motor control as a result of denervation, which causes denervated muscle fibres to die (Rowan *et al*, 2012). In the event that the fibres are reinnervated by axonal sprouting, the fibres will survive, but will be "slaved" to fire when the rescuing axon fires, even if this was not the case previously. In this way, fine motor control is lost with age, in a manner that does not occur in disuse atrophy, and it is thought microgravity atrophy (Brooks and Myburgh, 2012). In cachexia, the muscle mass loss arises from Inflammation and oxidative stress, derived from and driving mitochondrial dysfunction (Rosa-Caldwell *et al*, 2019). Additionally, many chemotherapeutic drugs are known to contribute to muscle loss and weakness, in both the heart and skeletal muscle (Tarpey *et al*, 2019; Pedrosa *et al*, 2023).

Of the two pathological models, aging and cachexia, cachexia is the closer to disuse atrophy, sharing the inflammation>ROS>apoptosis pathway (Chopard, 2009). Although sedentary lifestyle does lead to muscle loss on Earth, it is not typically treated by pharmaceutical intervention, but by exercise. However, the bedrest model of microgravity has been used to

assess the potential changes in pharmacodynamic and pharmacokinetic properties of drugs, but with limited ability to reproduce effects observed in actual microgravity, possibly due to the effects of microgravity and possibly due to the relatively low sample size of purely pharmacology related human studies (Gandia *et al*, 2005). Drugs used to treat muscle loss on Earth typically focus on the muscle of the heart as its weakening is most easily associated with death, understandably so. However, skeletal muscle treatments are somewhat less common. Overall, the strategy is to upregulate anabolic pathways as much as is sustainable, whilst dampening catabolic signalling. Sustainability is a major issue when considering tolerability and systemic effects. Stimulation of skeletal muscle β2 adrenergic receptors can produce beneficial effects in that tissue, but the heart also expresses these same receptors, leading to the possibility of arrythmia (Ryall and Lynch, 2008). One drug which has seen success in rodent models is allopurinol. Allopurinol is a Xanthine oxidase inhibitor, which results in a decrease, but not elimination of ROS production in skeletal muscle (Matuszczak *et al*, 2004). By removing additional ROS production seen in inactivity atrophy, the mitochondrial/ ROS apoptosis pathway and ROS mediated ubiquitination pathway can be avoided, resulting in improvement in muscle mass loss. Unfortunately, this does not treat the reduced muscle protein synthesis that occurs in disuse atrophy, only the increase in breakdown (Ferrando *et al*, 2017). However, signal pathways tend not to fit neatly into "good" or "bad". It is more accurate to consider the function of the pathway and the effect that a stable feedback loop involving multiple pathways might have. This requires a greater level of understanding of the timing and location of key mediators and executors within the pathways, and rapidly exposes gaps in our current understanding of how "canonical" pathways interact over time and with changing conditions (Winslow and Hall, 2019). Treatment of muscle wasting focused on the mitochondrial overproduction of ROS by mitoquinones has not shown beneficial effects (Morley, 2016),

possibly due to the total abolition of ROS signalling, which reduces antioxidant gene upregulation, without which, insulin response suffers.

The Rodent Research-6 mission conducted by NASA and space X -though not yet published- aims to use slow-release implants in mice to trial the asthma drug Formoterol as a potential counteragent to muscle atrophy in microgravity (NASA, 2018). Formoterol, like many asthma drugs, is a β2 receptor agonist that causes bronchodilation when taken at relatively low doses. For some time the potential for β2 adrenoreceptor agonists to act as beneficial modulators of skeletal muscle activity has been in doubt, but a recent study focusing on multiple different agonist drugs demonstrated that downregulation of degradation pathways occurred with Clenbuterol, but not other agonists, suggesting a biased ligand model response occurring during signal transduction (Wannenes *et al*, 2012). Formoterol has been identified to slow the progression of disuse atrophy in both rat models (Gómez-SanMiguel, *et al* 2016) and humans with spinal injury (Scholpa *et al*, 2019), making a good case for further investigation into adrenergic receptors as druggable targets.

1.5.2 Pharmaceuticals for the maintenance of glucose uptake

Another class of drug under consideration for treatment of insulin resistance is AMPK activators. Whilst it is true that there is strong evidence that AMPK activators improve glucose uptake in muscle (Coughland *et al,* 2014), it is also known that the role AMPK plays physiologically during starvation is both anti-anabolic and pro-catabolic for the alpha-1 and alpha-2 isoform respectively (Thomson, 2018). This combination of effects may make it an unsuitable candidate for upregulation to improve insulin response among astronauts specifically.

However, anti-diabetic agents are a popular field of study and as such there are many drugs and drug candidates that could prove suitable for the maintenance of glucose uptake in space. Whilst metformin is also an AMPK agonist, it is known to work through several pathways, not all of which have been identified (Sanches-Rangel and Inzucchi, 2017). It has been shown in rats that metformin is able to mitigate the fibre type switch that muscle undergoes as part of disuse atrophy and that this may be useful in microgravity (Mirzoev *et al*, 2023). In their review, JAXA, the Japanese space agency, identified AICAR as a potential therapeutic drug to counter the negative metabolic effects of microgravity on skeletal muscle, when administered alongside other PPARγ activators (Furukawa *et al*, 2021). AICAR activates AMPK by mimicking the activity of AMP, resulting in signalling activity similar to that seen during depletion of ATP (Višnjić *et al*, 2021). Studies involving AICAR application to C2C12 cells have shown that AICAR can increase mitochondrial biogenesis (Hinkle *et al*, 2022) but also inhibition of hypertrophy (Egawa *et al*, 2014).

1.5.3 Potential problems with pharmaceutical interventions

The main impediment to the establishment of successful pharmaceutical treatments for muscle atrophy is a lack of understanding of the signalling loops involved in maintaining the atrophic state despite changing conditions. Whilst the importance of decreased protein synthesis is agreed upon, the impact of potential increase of protein degradation remains disputed (Bodine, 2013). This makes selection of a druggable target difficult as it has been repeatedly shown that blanked downregulation of “catabolic” proteases or ROS do not yield the expected improvement in muscle mass or function (Bell *et al*, 2016). Upregulation of

anabolic targets such as mTOR and ERK1/2 could also prove problematic, as there is a reason that these mediators are under tight control by default and that is their ability to cause cancer if overstimulated. These problems may well be connected to the transient nature of the signalling changes that occur within muscle, which allows muscle to adapt rapidly to the environment and gives it it's plastic nature (Bruton, 2002).

Another problem with a pharmaceutical solution to muscle atrophy is the potential for side effects or reactions between drugs taken on a regular basis for muscle maintenance and drugs that may be taken only sporadically to deal with other issues. Protection of bone and muscle from inactivity by treatments which reduce myostatin are currently showing promising results (Lee *et al*, 2020), however there have been many attempts to protect muscle by manipulation of myostatin and it has been a recurring problem that there are serious off target effects, mainly in soft tissue such as bleeding and erythema (Suh and Lee, 2020).

1.6 Investigation of possible countermeasures to deconditioning in cell culture models

1.6.1 Media conditions

The most common practice is to grow C2C12 cells in a high glucose DMEM amended with L-glutamine, a penicillin-streptomycin mixture and 10% Foetal Bovine Serum (FBS). This media is optimized for rapid proliferation of myoblasts, a large number of which are required in order to fuse into myotubes. Fusion is achieved by switching to a differentiation media that switches 10% FBS for 2% Horse serum. The reduction in serum concentration stimulates the myoblasts to differentiate. Though it would be ideal to differentiate myoblasts on a patterned or pliable hydrogel substrate, these are not yet at the point of common commercial availability.

Whilst it is possible to grow C2C12 myotubes on low glucose media, doing so would eliminate one of the main benefits of the model which is the rapid speed at which proliferation occurs (Elkalaf *et al*, 2013). Reducing the growth speed reduces the ease with which stocks can be built up and also reduces the flexibility in experiment planning that fast growing cells have due to their abundance. Additionally, by saturating glucose transporters- specifically constitutively active GLUT1- from growth, through differentiation to experiment the closest thing to a nutritional and environmental steady state can be obtained, minimising variability in uptake that might be introduced by having limited availability of glucose (Chadt and Al-Hasani, 2020).

1.6.2 Tracer use

In human volunteers a glucose clamp is the gold standard for determining the uptake of glucose in response to insulin. In cell culture applications, glucose tracers are used to determine the uptake of glucose into the cell. A suitable tracer should have the same uptake kinetics as glucose whilst also being in some way distinguishable or detectable from "endogenous" glucose. Traditionally this has been achieved though radiolabelled glucose, usually C14 at the 1^{st} or 6^{th} carbon atom. However, substitutions at the 1^{st} carbon are subject to metabolism by the pentose cycle and so do not match values provided by 6^{th} carbon substitutions (Katz and Wood, 1960). To get around this issue, non-metabolizable radioactive tracers such as Fludeoxyglucose can be used, which are detectable by the radiation they emit (Ashraf and Goyal, 2023). Using these tracers in a cell culture model significantly simplifies the resulting analysis as there are fewer compartments in which the tracer might become sequestered, confounding the results. Similarly, a non-metabolizable tracer is not subject to escape from the compartment of interest as "waste" metabolites in the same way as metabolizable tracers, which is a larger problem in the tracing of amino acids.

It is desirable to minimise the requirements for radioactive compounds that researchers are exposed to and so a stable (non-radioactive) isotope can be used and rather than detection by scintillation counter, the sample is fractionated on a mass spectrometer and the heavy stable isotope is detected in the fragmentation products. When the background concentration of this isotope is accounted for, the uptake of glucose can be determined using the content of the isotope contained within the sample (Kim *et al*, 2016). Until recently, 2-deoxy-D-glucose (2-DG) has not been used as a glucose tracer in whole animals, due to its propensity to inhibit glycolysis at high concentrations. Despite this, it is a good candidate for use as a short-term glucose tracer as it follows the same uptake kinetics as endogenous glucose, its non-metabolizable, has no pathway out of the cell and can be discriminated from glucose on mass spectrometer. By working with a small amount of muscle in a cell culture setting it should be possible to use a dose of 2-DG that is low enough that it does not inhibit glycolysis, but high enough to be detectable, unlike in whole animals (Ralser *et al*, 2008).

1.6.3 Electric Pulse stimulation

Electric pulse stimulation (EPS) has been used for at least 134 years as method to study contraction in excitable tissue such as skeletal muscle *ex vivo* and *in vivo (*Sewall *et al*, 1888). The electrical stimulation of cultured myotubes is considerably more recent, with the first culture of chick muscle in 1946 by White (White, 1949), who demonstrated that chick cardiac muscle could be cultured and sustained on media with a defined composition. This differed from the normal practice at the time, which was to use high serum solutions, leading to cultures almost entirely consisting of fibroblasts. The culture of skeletal muscle enabled experimentation with myotubes independently of donor animals, but to our knowledge, the first instance of cultured muscle cells being stimulated by external electrical input was not until

1971, though non-electrical methods of stimulation, such as addition of acetylcholine, bathing in potassium containing solution, as well as electrical stimulation had already been developed at this point for use in isolated muscle tissue (Fischback *et al*, 1971; Rudeanu and Botez, 1950). These methods exploit the characteristics of excitable tissues in order to cause depolarization, leading eventually to contraction.

EPS in cell cultures is carried out by passing a current, termed a pulse train, through the cells, via the media which induces an action potential in the excitable cells (Kaji *et al*, 2010; Fish *et al*, 2009). Contraction modality (twitch vs tetanic) can be controlled by modulation of the pulse train's frequency and duration to achieve the desired contraction rate. Commercial equipment for the purpose of electrical stimulation is available, however this is not always the case, and many laboratories use self-built equipment, which can accommodate conditions not covered by pre-made equipment (Nikolic and Aas, 2018). Equipment choice is an important consideration. Different materials can be used for the electrodes which will be placed into the culture media, transferring current to the cells. Carbon is often used in cell culture settings, where long term stability is not required. Other materials such as platinum, gold, titanium or agar-salt bridges have been used, with varying levels of success (Meng *et al*, 2022; Brevet *et al*, 1976). The instability of carbon electrodes does not come from the degradation of the electrode itself, but rather accumulation of cell waste materials in the porous structure of the graphite electrode (Ahadian *et al*, 2012) necessitating frequent cleaning. Despite being relatively inert, platinum electrodes *are* subject to degradation and the products from this corrosion has been shown to exert cytotoxic effects on cells *in vitro* (Wissel *et al*, 2018).

Typically EPS involves muscle cells cultured on a cell culture treated plastic plate, paced for the desired duration in order to induce contraction. The duration of EPS depends highly on the

experiment being performed, but also on the cell type. For example, human primary cells in culture have relatively poor contractility under standard cell culture conditions. The two main rodent muscle lines used are L6 (rat) and C2C12 (mouse) cells and they differ significantly in their expression of metabolic and contractile genes, as discussed by Abdelmoez *et al* (2020) who highlight the greater contractile ability of C2C12 cells, whilst L6 cells offer a greater metabolic response to EPS. Increasing the voltage of the pulse train is one way to improve the rate of recruitment of cells to contraction (Tamura *et al*, 2020). However, doing so will increase the heat generated in the media. Furthermore, not all cell types will contract immediately upon the passing of current. A pre-stimulatory period is often required in order to induce the formation of fully functional sarcomeres in newly differentiated C2C12 myotubes for example (Fujita *et al*, 2007). Similarly, it is accepted that cultures of human myotubes will not spontaneously contract in isolation (Guo *et al*, 2013). Sarcomeres formed in this way are dependent on the continuous input of current for their maintenance and will quickly disassemble if electrical input is withdrawn (Fujita *et al*, 2010). Alternatives to electrical induction of sarcomere formation and contraction include light -based activation of contraction (Sakar *et al*, 2012; Sebille *et al*, 2017). Though increasingly popular, this requires transfection of cells as well as a longer differentiation time before contraction can occur- 10 days as opposed to 5. Though it is a vital part of the EPS procedure, it is also a confounding factor which needs to be accounted for when examining particular or early timepoints.

Factors that must be considered in the design of an EPS experiment is that during the contraction protocol, cells will be secreting waste metabolites as well as signal molecules into the media (Sciancalepore *et al*, 2022; Nedachi *et al,* 2008). If a chronic EPS protocol is being applied, then the culture media will need to be changed during the experiment in order to

replenish the nutrients in the media. Both of these introduce variability, especially if continuous stimulation is a requirement. The removal of the pulse train removes many of the downstream signals which induce muscle cells to mature in their differentiated form (Fujita *et al,* 2010). As such, a "standard" EPS setup is limited in the duration or type of contraction experiments it can perform. The time of harvesting of the media and cells depends on the endpoint being measured and its expected response time to the applied stimulus. For example, many signalling responses can be seen during and immediately after contraction ends, whilst changes in protein can occur over a period of hours post contraction (Tarum *et al*, 2017).

1.6.4 Potential limitations

Human and animal muscle cell lines are also available for study of skeletal muscle *in vitro*. They have many advantages such as lower cost, fewer ethical concerns and greater specificity for effects intrinsic to the cell type being studied, rather than systemic effects. The drawback associated with these models is the inconsistency of culture conditions between groups, the lack of systemic effects as well as some artefacts unique to tissue culture.

These artefacts typically arise from differences that exist between real microgravity and simulated microgravity. In real microgravity there is close to zero spontaneous convection (separation by density) and no shear stress imparted to cells by the surrounding media. Both of these are induced in simulated models of microgravity, since rotation causes cell media to move around the central point of rotation (Wuest *et al*, 2015; Leguy *et al*, 2017). In effect, RPMs (Random Positioning Machines), clinostats and RWVs (Rotating wall Vessels) only change the orientation of cells so that they cannot biologically perceive or respond to gravity over

time. In a physical and instantaneous sense, gravity still applies to the cells (Herranz *et al*, 2013). Additionally, it is not possible to perform microscopy or EPS on cells that are in an active RPM. Magnetic levitation can also be used to simulate microgravity on cells and small animals such as frogs (Berry and Geim, 1997). Whilst this does reproduce many of the changes seen in cells exposed to real microgravity, there are concerns that it is the strong magnetic field and not the levitation that induce the changes, leading many to question the representativeness of results obtained by this method (Moes *et al*, 2011). Ultimately it is necessary to verify the findings of simulated microgravity studies in real microgravity where possible, regardless of the simulation method. *In vitro* studies using C2C12, P19, L6E9 cell lines among others, have provided insight into the relationships between microgravity, metabolic disruption and exercise, paving the way for more effective countermeasures both on earth and in space (Nickolic *et al,* 2012).

In the C2C12 cell line used in this project the major limitations are threefold. Firstly, C2C12 cells require pre-stimulation by EPS in order to form sarcomeres able to contract in a manner that represents *in vitro* contraction patterns (Nedachi *et al*, 2008). This introduces the possibility that EPS stimulated sarcomeres are in some way different from normal sarcomeres which contract in response to neuromuscular junction derived excitation. That said, C2C12 have seen successful use as a model of human exercise and insulin response. (Nikolic *et al*, 2012; Carter and Solomon, 2018; Wang *et al,* 2016; Fraser *et al*, 1993).

Another potential issue is the physiological difference between mice and humans. Unlike rodents, humans do not express the type 2 b fast muscle fibres, instead expressing an intermediate fast fibre referred to as type 2x/d. In culture, c2c12 cells are capable of forming mixed fibres as in *vivo* bundles do. Because fibre type is closely aligned with oxidative metabolism, the culture conditions can greatly affect the fibre type being experimented upon,

which should be taken into consideration when planning experiments (Hanke *et al*, 2011; Khodabukus and Baar, 2014).

The third problem of a cell model of microgravity is that under cell culture conditions the cells are expressing a phenotype that is in many ways like foetal tissue (Tesseraux, 1987). An example of this in the C2C12 line is the expression of the gamma subunit of the nicotinic Acetylcholine receptor, rather than the epsilon subunit (Cetin *et al*, 2019). It is understood that there are differences between adult and foetal muscle in response to fatigue, but other differences may introduce inaccuracies when extrapolating results to adult human muscle function (Racca *et al*, 2013). This is a limitation of many cell models, but it could also be a useful indicator for culture or treatment conditions that shift the overall phenotype towards an adult expression mode.

Finally, although ground-based simulation of microgravity is valuable because it improves accessibility to a microgravity environment, it is not a full reproduction. This could cause issues with formulation of drug countermeasures to microgravity disuse wasting, due to changes in how cells interact with drugs (Kohn and Hauslage, 2019) and isoform stability in microgravity, owing to changes in storage conditions, mainly lack of gravity and heightened radiation exposure (Mehta and Bhayani, 2017).

1.7 Aims and hypothesis

Exercise is a known countermeasure to disuse induced atrophy and related reduction in glucose uptake. The aim of this thesis is to establish a viable C2C12 model of disuse atrophy in

order to further the study of interventions for the treatment of disuse related reduction in glucose uptake and muscle mass. To do so, there were several questions that needed to be answered.

Chapter 2

In order to reliably track changes in glucose we needed to know if there was a dose of 2-DG that could be applied for 24 hours in both the presence and absence of EPS that would be detectable on mass spectrometer, but not so high as to inhibit glycolysis. We hypothesized, based on linear uptake achieved by bathing excised muscle in 2-DG containing media (Hansen *et al*, 1991), that 200µM 2DG would be a high enough dose to be detectible on mass spectrometer, yet low enough that glycolysis would not be disturbed, as measured by the export of lactate to the media. Additionally, we aimed to prove that in our hands, C2C12 cells would respond to EPS in a manner that was consistent with the wider literature. We hypothesised that as well as an increase media lactate in response to EPS, there would be changes in mTOR, 4EBP1 and P70 phosphorylation indicative of either exercise induced suppression of protein synthesis or increased synthesis, given the low resistance of the exercise being emulated by the chosen pulse train of 11.5V, 2ms pulse duration at 1Hz.

Chapter 3

One of the major challenges associated with the treatment of disuse atrophy and associated insulin resistance is prescribing "enough" exercise. Having established that C2C12 cells would respond to EPS in a manner consistent with the wider literature and that we could track glucose uptake with 2-DG without disrupting glycolysis, we aimed to determine the point between continuous and no EPS at which there were diminishing returns on glucose uptake or protein synthesis measurements. We hypothesized that, as linear uptake had been established

over 24 hours of continuous EPS, the glucose uptake response would be directly proportional to the time spent contracting. We expected that changes in pro-synthesis signalling may reach maximum intensity or saturation after only a few hours of EPS.

Chapter 4

Two of the major pathological hallmarks of disuse atrophy at the cellular level are elevated cytoplasmic calcium and reduced physical activity. In order to recreate the downstream phenotype generated by chronic exposure to these conditions we aimed to impose them through the addition of CPA and Blebbistatin. CPA is a SERCA pump inhibitor. By inhibiting the re-uptake of calcium to the sarcoplasmic reticulum we intended to raise the cytoplasmic concentration of calcium. Blebbistatin is an inhibitor of myosin II ATPase, which binds to the ATPase and locks it in the tension free state, preventing actin-myosin bridge crawling. We hypothesize that with the application of both of these drugs, conditions would be created that would lead to reduction of glucose uptake and changes in signalling that favoured breakdown of contractile elements rather than synthesis.

Chapter 5

Having established a model in which elevated calcium and lack of contraction prevented glucose uptake and protein synthesis signalling changes in response to EPS, we aimed to test the reversibility of the simulation of disuse imposed through CPA and Blebbistatin. We aimed to reverse this by application of drugs that would specifically act in a manner counter to CPA and Blebbistatin. AICAR, an AMPK stimulator would act downstream of the -now permanently inhibited- contractile pathway, whilst dantrolene would prevent a CPA induced rise in cytoplasmic calcium by stabilising the RyR in the closed conformation. We hypothesized that this would only partially restore glucose uptake.

Chapter 2: Validation of the measurement of 2-Deoxyglucose uptake in C2C12 with EPS

2.0 Introduction

Inactivity is linked to the development of insulin resistance (Shur *et al,* 2021). A reduction in response to insulin throughout the body can have many effects, but in muscle the primary effects are a reduction of growth hormone signalling through the IRS1- mTOR- P70 as a result of overactivation of mTORC1 and reduction in mTORC2 (Ong *et al*, 2016). This results in reduced muscle mass and strength through removal of insulin associated inhibition of proteolytic pathways (Wang *et al*, 2006) as well as reduced glucose uptake (Merz and Thurmond, 2020). The problem of insulin resistance derived from disuse is one of growing severity, as more of the population take up jobs that involve sedentary habits (Khan *et al*, 2020). As well as reducing response to insulin, muscle disuse itself removes many of the signals that act to maintain muscle mass and the recruitment of GLUT4, therefore astronauts- a cohort that would otherwise be physically fit- are also at risk from the effects of muscle disuse on strength and glucose uptake. In astronauts, the symptoms of muscle disuse could lead to physical weakness that could result in personal danger and the potential failure to complete mission objectives on mars or the moon.

2.0.1 Investigating muscle function and metabolism *in vitro*

A skeletal muscle model of disuse intended for use in measuring the effect of pharmacological and exercise intervention would be useful. Though the results of rodent cell line studies cannot be extrapolated 1:1 to human beings, they are a useful and accessible platform to test initial assumptions. The C2C12 cell line has previously been used as a platform to measure exercise response (Nedachi *et al*, 2008). Given that this cell line can change its glucose uptake in response to physical activity and to a lesser extent insulin, as well as undergo hypertrophy (Tsukamoto *et al*, 2019) it has the

necessary functionality required in theory to act as a model for disuse. To do this, it had to be shown that in our hands the cells respond normally, as without a normal initial response to EPS, it would be difficult to prove that inactivity had been achieved. In order to induce the formation of functional sarcomeres, C2C12 cells must be pre-stimulated with EPS, so that when experimental EPS is applied the cells will be capable of contraction from the outset (Fujita *et al*, 2007). In order to minimise the risk of overshadowing changes induced by the experimental EPS with changes caused by pre-stimulation, the pulse train conditions were kept the same. As the rate of glucose uptake in cells expressing multiple GLUT isoforms can vary at sub maximal glucose concentrations, high glucose DMEM was chosen as the basis for cell culture media (Chadt and Al-Hasani, 2020).

2.0.2 2-Deoxyglucose as a glucose tracer

To measure the uptake of glucose over 24 hours we elected to use 2-DG (2-Deoxyglucose) as a tracer because it was non-metabolizable, had no route of exit from the cell after phosphorylation and could be detected on mass spectrometer. 2-DG is an analogue of glucose bearing a substitution on the second carbon atom. The expected hydroxyl group found on D-glucose is instead replaced by a hydrogen atom (Aft *et al,* 2002). What remained to be seen was the exact concentration at which 2-DG could be detected yet would not interfere with glucose uptake by inhibition of HKII (Hexokinase II), as well as other elements of glycolysis. A diagram of the inhibition of glycolysis is presented below (**Figure 2.1**), adapted from Zhang *et al,* 2014. From the outset we

determined that changes in media lactate from baseline in the absence of EPS would be evidence of disruption of glycolysis by excessively high concentrations of 2-DG.

2.0.3 EPS as an exercise model

Whilst there have been many papers published in recent years concerning the use of EPS as an exercise mimetic, we are still some ways from full understanding how pulse train parameters map to changes seen in muscle. For example, whilst it is often said that low frequency EPS is mimetic of endurance exercise and high frequency EPS is mimetic of resistance exercise, the boundary between high and low frequency is indistinct, with some claiming low frequency exists between 1 and 20Hz, whilst others say 1-5Hz (Lautaoja *et al*, 2023). Cell type and origin can also play a role in response to EPS, as though 30Hz would be considered "high frequency", human primary muscle cells exposed to EPS at this frequency did not show increases in mTOR and P70 phosphorylation, when at 60Hz they did (Mettler *et al*, 2018). Additionally, the duration of EPS seems to have some bearing on whether the muscle responds in a manner more consistent with resistance or endurance type exercise. Though both modalities have been demonstrated to increase glucose uptake in C2C12 cells (Lautaoja *et al*, 2021; Burch *et al*, 2010), signalling changes relating to protein synthesis are more difficult to interpret, with some studies showing increase in protein synthesis and other showing reduction. Scheler *et al* (2013) report that in C2C12 myotubes they found an increase in P70 phosphorylation after 24hr high frequency (30Hz) EPS, but not after low frequency (5Hz). However, Tamura *et al* (2020) report -also in C2C12- depression of P70

phosphorylation immediately after low (2hz) and high (66Hz) frequency stimulation. These differences could stem from small differences in pulse train setting that over hours of EPS have a cumulative effect, but because of the lack of repeated use of "established" pulse train settings, it is difficult to compare outcomes between studies with a high degree of certainty (Nintou *et al*, 2022). Alternatively, differences could stem from the intensity of the contraction relative to how developed the contractile elements within the myotubes are. Tarum *et al,* 2017 showed that in human myotubes immediately post EPS (12V, 2ms, 1Hz), phosphorylation of mTOR increased alongside a matching increase in 4EBP1, yet P70 remained unchanged though hypertrophy did occur, something that they attributed to time from contraction. This demonstrates the importance of timepoints when taking readings during and after contraction *in vitro*. We have chosen to harvest cells immediately after contraction for 0.5, 4, 8 or 24 hours in this chapter.

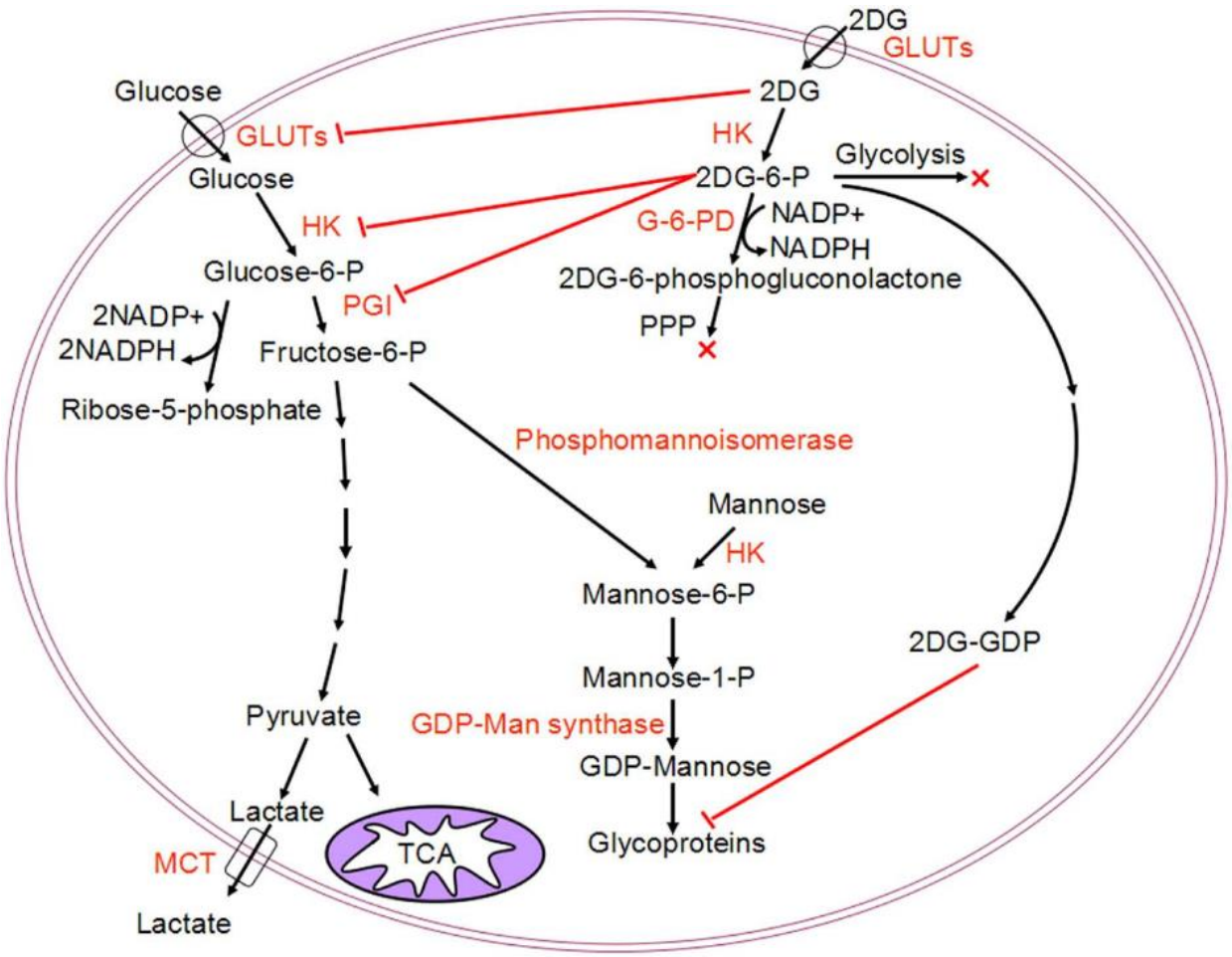


Figure 2.1: Diagram of the inhibitory effect of 2DG on glycolysis and by extension glucose uptake resulting in downstream depletion of lactate. Competitive inhibition of hexokinase II increases with accumulation of 2DG6P thanks to its inability to be enzymatically isomerized.

2.0.4 Aims and hypothesis

In this chapter our aim was to demonstrate that there was a viable dose of 2-DG that could be used to trace glucose in the unstimulated (basal) and EPS stimulated state, without disrupting glycolysis. This was to be determined by linearity of glucose uptake as well as by confirming that there was no difference in lactate output between control cells and cells provided with 2-DG. We hypothesized that 200µM 2-DG would be a suitable dose, however this proved not to be the case, so the dose was revised down to 25µM.

Additionally, we aimed to show that 24 hours of EPS at 11.5V, 2ms, 1Hz would increase lactate as glycolysis would increase to provide additional ATP for contraction. This would provide evidence of normal metabolic response to EPS, along with increase in glucose uptake. We hypothesized that in line with literature examples there would be a 2-3 fold increase in glucose uptake in response to EPS. Lastly, we aimed to show that in our hands EPS would induce changes in phosphorylation protein synthesis related targets mTOR, P70 and 4EBP1. We hypothesized that as we harvested cells immediately post contraction that we would see suppression of mTOR and P70 phosphorylation alongside elevation of 4EBP1 phosphorylation.

2.1 General Methods

2.1.1 Material preparation

Dulbeccos Minimal Eagle Media (Sigma, D6546) amended with 10% FBS (Sigma, F9665) and 1% L-glutamine (Sigma, G3126), pen-strep (Sigma, P4333) was used for growth and freezing of C2C12 mouse myoblast cells (ATCC, CRL-1772). Differentiation media was prepared to the same specification but with 2% horse serum (Sigma, H1270) replacing 10% FBS. Corning tissue culture treated T75 flasks were purchased from Sigma as were cell scrapers. Corning 6 and 96 well plates were also purchased from sigma. For cell harvesting, solutions of 75% methanol in water and homogenisation buffer (HB) were used for cells intended for Mass spectrometer analysis of 2DG uptake and immunoblotting, respectively. HB consisted of 7.88g Tris-HCl, 0.372g EDTA, 0.380g EGTA, 2.16g β-Glycerophosphate and 2g NaF per litre.

A working stock of 2-deoxyglucose (sigma, 111980050) was prepared. Actrapid Insulin was purchased from Novonordisk.

For western blotting and immunofluorescence imaging antibodies were purchased from cell signal unless specified otherwise. Targets were, GAPDH, phospho-MTOR (ser2448), Phospho-4EBP1 (Thr37/46), Phospho-P70S6k (Thr389). Gels were run in 1X XT MOPS (Bio-Rad, 1610788). Per litre, transfer buffer was made up with 3.03g Tris, 14.4g glycine and 200ml methanol and 800ml dH_2O. TBST was used for membrane

washes and for blocking and antibody solutions (8g NaCl, 2.42g Tris base, 1ml Tween-20 per litre).

2.1.2 Cell culture

C2C12 cells were seeded to T75 flasks and grown to 80-90% confluence at 37°C, 5% CO_2 in 10ml growth media. Cell splitting was performed using two washes of Hank's Buffered salt solution (HBSS) (sigma, H9269), followed by detachment using trypsin replacement TriplExpress (Gibco, 12605010). Stock building was achieved by freezing in Recovery freezing solution (Gibco, 12648010) and a Mr frosty (Thermo Scientific, 5100-0001) freezing apparatus followed by storage in liquid nitrogen. As cells would rapidly fill a T75 flask, rather than use a haemocytometer, a flask at 90% confluence with myoblasts would be split into three further T75 flasks. These receiving flasks would then themselves reach 90% confluence 24-48 hours later. Cells for experimental use were seeded to 6 well plates (corning, 3506) at passage number 10-12. Once cells had reaches 80-90% confluence they were switched to differentiation media and allowed to differentiate for 5 days. Throughout the growth and differentiation process media was changed every 2 days. Cells were always fed 24 hours before beginning experimental conditions in order to establish a consistent baseline metabolic state.

2.1.3 Cell pacing by electric pulse stimulation

EPS was performed on fully differentiated myotubes using an Ionoptix C-Pace EP (IonOptix, Milton, MA, USA) connected to two 6-well compatible C-dishes, also from Ionoptix. Before each use, the electrodes were drained of waste build up and sterilized

with ethanol before rinsing with dH_2O. The pulse train settings were as follows: 11.5V, at a frequency of 1Hz with each pulse lasting 2ms, informed by previous studies which used the same settings and saw release of exercise associated cytokines (Evers-van Gogh *et al*, 2015). Similar pulse train settings also caused uptake of glucose (Nikolic *et al*, 2917). A volume of 2ml media per well was sufficient to ensure electrodes were submerged. Cells were subjected to 1 hour of pre- stimulation to allow time for functional sarcomeres to form. Pre-stimulation used the same pulse train settings as the experimental EPS, 11.5V, 1Hz, 2ms. After this, cells received constant pacing for either 0 (control),0.5, 4, 8 or 24 hours. Control plates (unstimulated) were kept for 8- and 24-hour timepoints to control for diurnal changes to glucose metabolism.

At the end of the experimental pacing, cells were harvested by removal of media – retaining 1ml-, washing twice with PBS and then scraping into either HB or 75% methanol.

2.1.4 Microscopy

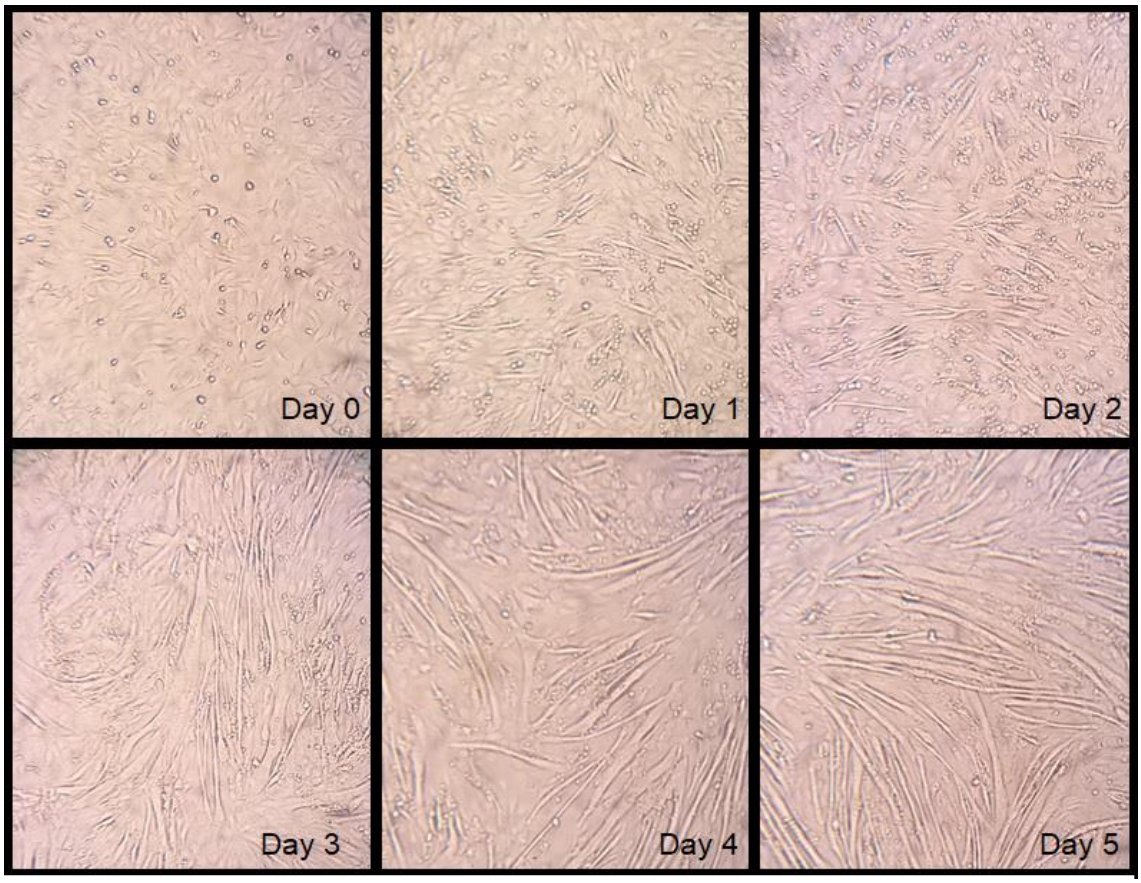


Figure 2.2: Differentiation of c2c12 myoblasts to myotubes. Serum withdrawal is achieved by switching from 10% FBS to 2% Horse serum, with media being changed every second

Bright field microscopy at 10X magnification on a Ziess ID 03 microscope was used to inspect C2C12 growth and differentiation (**Figure 2.2**). For immunofluorescence microscopy as well as real time video footage capture, an EVOS M7000 (Thermofisher, AMF7000) imaging system at 20X magnification was used. Real time imaging in light microscopy mode was used to confirm the contraction of myotubes during the application of EPS. A video demonstrating contraction can be seen at the following link:

https://uniofnottm-my.sharepoint.com/:v:/g/personal/euan_kirkpatrick_nottingham_ac_uk/ERiviBvnDJxNhOSpEb-kdBcBzT5cJH-N1Zkp2tORvm38sQ?email=mdzii%40exmail.nottingham.ac.uk&e=Kke1W3

2.1.5 Western Blot

Sarcoplasmic fraction for western blot was isolated by centrifugation of C2C12 cells lysed in HB at 11,000g for 15 minutes at 4°C. Protein present within each sample was determined using a nanodrop one microvolume UV-vis spectrophotometer (Thermo Scientific, ND-ONE-W) at 280nm. Samples were then made up with 4X laemmli with 10% β-Mercaptoethanol in a ratio of 1:3 buffer: sample, with 10µg protein being loaded to each well.

Quantitative western blotting was employed to detect changes in the proteome over the experimental timepoints. A precast Bio-Rad XT 12% Bis-Tris (Bio-Rad, 3450119) gel was loaded with sample after submerging in running buffer, with 1 lane dual colour chemiluminescent ladder (Bio-Rad, 161-0374) and the last two lanes with "All blue" colorimetric protein standard (Bio-Rad, 161-0373). After running for 70 minutes at 170V in a "Bio-Rad criterion Cell" gel running tank (Bio-Rad, 4206272), gels were washed and transferred to PVDF membranes by electrophoresis on ice in a "Bio-Rad criterion Blotter" tank (Bio-Rad, 1656024). Membrane blocking was in 3% FBS in Tris buffered solution with Tween20 (TBST). Primary incubation was overnight at 4°C at a concentration of 1:1000 in a TBST solution with 2.5% w/v BSA. Secondary antibody

incubation was for 1 hour at room temperature at a dilution of 1: 2000 with 2.5% w/v BSA. The concentration of Strep conjugate in the secondary mixture was 1:20,000.

Imaging was on a Fujifilm LAS 3000 and densitometry analysis was performed in image J (Schneider *et al*, 2012). Statistical analysis and presentation of data was performed in GraphPad Prism version 9.1.1 (221) for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. One-way ANOVA and mixed model ANOVA were used as appropriate for data analysis.

2.1.6 Lactate Assay

The concentration of lactate in retained spent media was measured by assay. The principle of this assay is the stochiometric conversion of lactate to pyruvate and $NADH^{+}$, the latter of which will absorb light at 340nm. This reaction is catalysed by lactate dehydrogenase in the presence of NAD (Sigma, 10127965001) in a buffer of 0.4M hydrazine and 0.5M glycine. Absorbance is measured before addition of lactate and then after 30 minutes of incubation at 37°C, with interpolation of the resulting absorbance with a standard curve of known lactate concentrations. A Molecular devices SpectraMAX 190 was used for measurement of absorbance on Corning 96 well plates. Data analysis was performed on results normalised against the control group for the respective experiment.

2.1.7 GC-MS measurement of glucose uptake

Cells that were harvested and scraped in 75% Methanol were then prepared for mass spectrometry. Cell fragments were spun down and resulting supernatant collected. Supernatant was spiked with C13-2DG6P as an internal standard. Spiked supernatant was evaporated with nitrogen flow using a Turbovap (Biotage) and the sample plus standard derivatized by addition of Methoxylamine chloride, with incubation at 90°C for 1 hour in order to oximize the sample. The derivatization was completed by silylation by addition of equal volume (75µl) of BSTFA followed by a further hour of incubation at 90°C for 1 hour.

2.2 Experimental Design

2.2.1 Glucose uptake in the absence of stimulation

To determine the optimal concentration of 2DG for detection on mass spectrograph without impacting glucose metabolism, 2DG uptake was measured at 0, 0.5, 4,8 and 24 hours for 200mM 2DG and later repeated for 25mM. The concentration of 2DG6P was measured by gas chromatography mass spectrometer after separation using a DB-5 (Agilent) polysiloxane column.

2.2.2 Glucose uptake in response to stimulation

Cells were pre-stimulated (in the absence of 2DG) for 1 hour and then immediately after provision of 2DG, EPS was carried out, with cells harvested at the same timepoints as previously. GC-MS was then used to quantify the cytosolic 2DG6P concentration as an indirect measure of 2DG and therefore glucose uptake.

2.2.3 EPS induced changes in glucose metabolism, MPS and MPB

Once the formation of myotubes had been confirmed by bright field microscopy a pre-stimulatory session of EPS was applied for 1 hour to induce the formation of functional sarcomeres. The settings for this EPS were as follows: 11.5V, 2ms, 1Hz. The effect of a further 24 hours of EPS on MPB/MPS was determined by subjecting C2C12 myotubes to EPS for 0.5, 4, 8 and 24 hours immediately after addition of 25uM 2DG to each well.

2.3 Results

2.3.1 Glucose uptake in response to 2-Deoxyglucose, with and without EPS

To establish a measurement of basal glucose uptake, the rate of glucose uptake in an unstimulated well containing myotubes was measured as described above. For cells provided with 200µM 2DG it was found that uptake remained linear only across the first 30 minutes before rate of uptake slowed (as shown in **Figure 2.3**). With linear uptake persisting until 0.58µM 2DG, it was decided to lower the total concentration to 25µM.

This lower dose of 2DG was expected to produce a linear uptake across the full 24-hour timespan, given that inhibitory concentrations would not be reached.

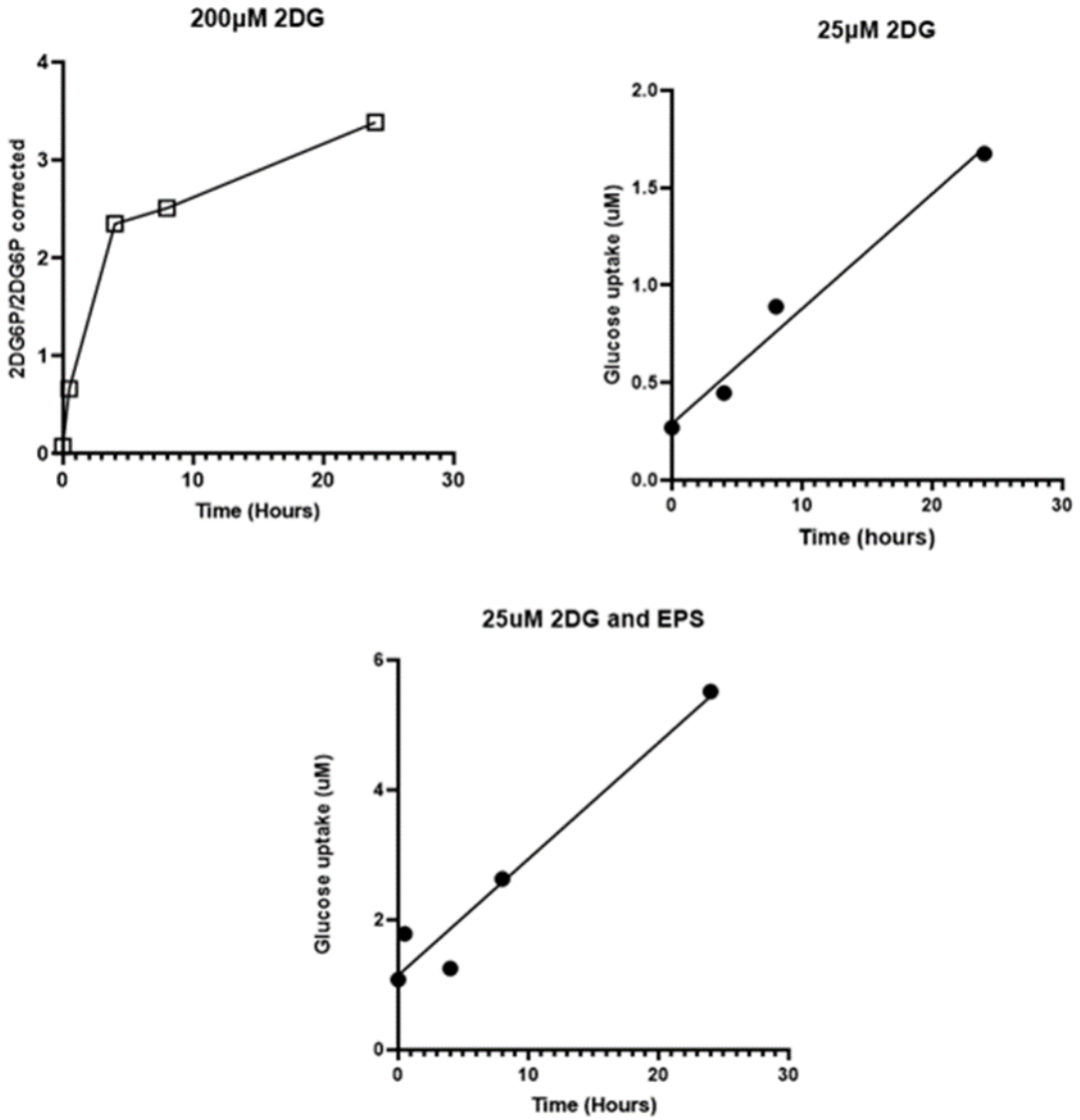


Figure 2.3: Uptake rate of 2DG at 25 and 200 µM in the absence and presence of stimulation**.** After treating with 200µM 2DG, cells were harvested at 0.5, 4, 8 and 24 hours. Linear uptake ended after approximetely 58µM 2DG had been taken into the cells. Uptake is displayed as the ratio of 2DG6P: corrected 2DG6P, where the corrected 2DG6P value accounts for the discrepancy between the 2DG6P standard and the 13-C-2DG6P internal standard. This proved necessary as some direct values of uptake in µM returned negative values. Cells treated with 25 µM 2DG showed linear uptake in the absence and absence of EPS.

2.3.2 Lactate output in response to 2-Deoxyglucose

Neither 200µM or 25µM of 2DG produced a significant change in media lactate concentration at the 24-hour timepoint (**Figure 2.4 A** and **B**). Whilst 25µM 2DG did not cause a change in media lactate at any timepoint, 200 µM produced an increase of 12.2 and 17mM when compared to baseline at 30 minutes and 4 hours ($p= 0.0121$ and 0.0038 respectively). This represents a greater than 2-fold increase at each timepoint.

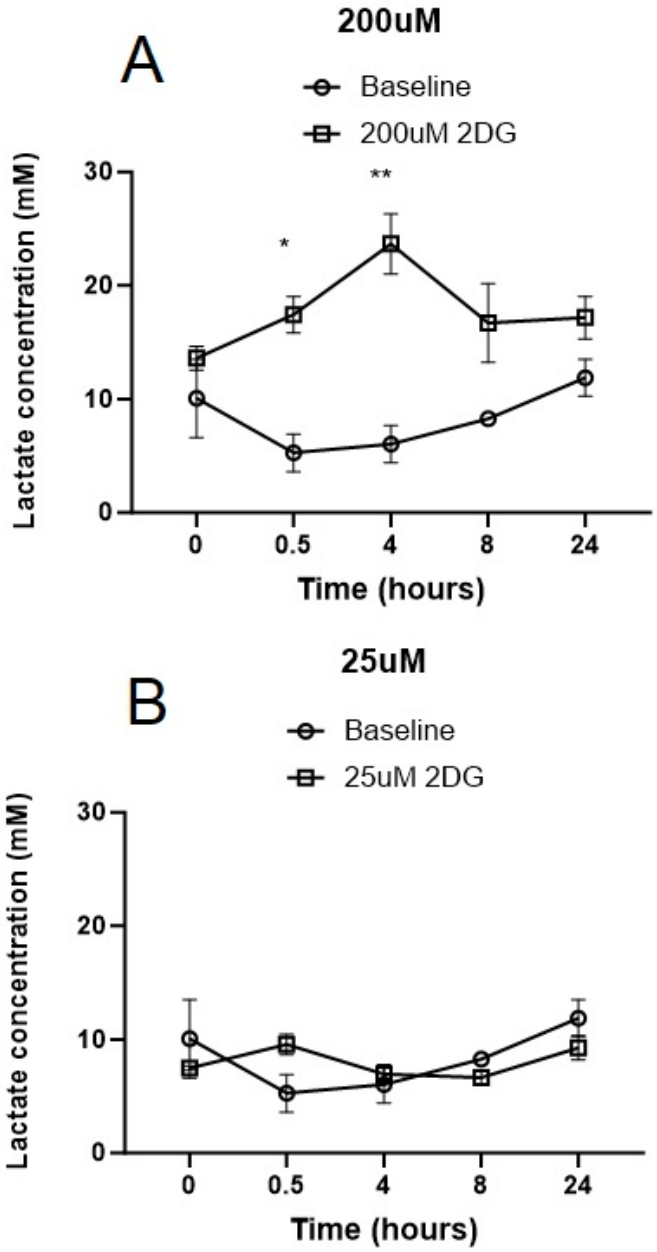


Figure 2.4: Change in absolute media lactate concentration for cells treated with either 200µM 2DG (A) or 25µM 2DG (B). Error bars represent Standard error of the mean. N=6 for 200 and 25µM series and n=3 for the baseline series .

2.3.3 Effect of insulin on lactate output

In the presence of insulin, media lactate concentration was elevated from the time of introduction of insulin and remained elevated until 24 hours. (**Figure 2.5**). Despite a decrease in lactate output at the 0.5 hour timepoint, the passage of time did not significantly change lactate output within the baseline cells.

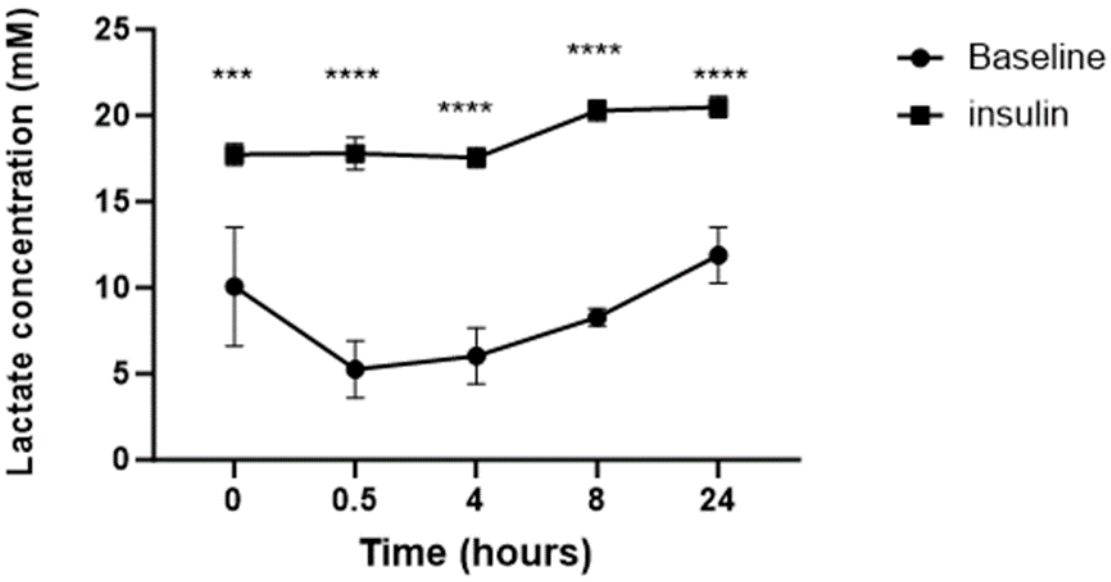
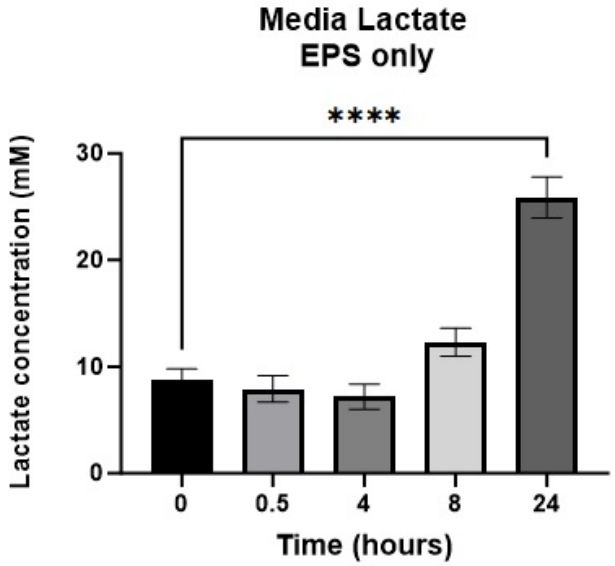


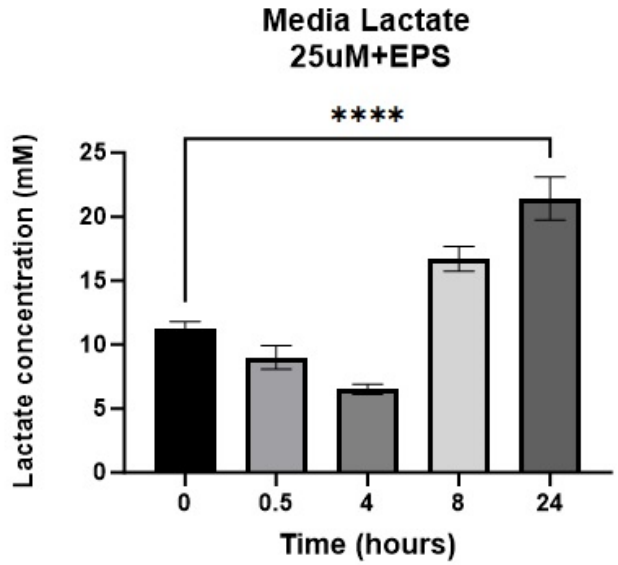
Figure 2.5: A: Absolute change in media lactate of cells treated with 2DG and insulin. Error Bars represent the standard error of the mean. N=6. Significant difference between insulin treated and untreated cells was observed. ***= p=0.0002. **** = p<0.0001.

2.3.4 The effect of EPS on lactate output

Electric pulse stimulation was carried out over a 24-hour period and media lactate concentration measured by assay. The effects of EPS on 25µM 2DG first become apparent at the 8-hour timepoint, with a high degree of significance (P= 0.0004) (**Figure 2.6**). This trend continues, with media lactate peaking at 24 hours of stimulation, represented by a 2.6-fold increase in lactate concentration when compared to 0-hour control.

When compared to an EPS only time course (**Figure 2.6**) there was little difference between EPS only and 25µM 2DG + EPS. Though the 2DG treated cells showed lower values at 24 hours there was no significant difference between treatments. The main effect of 25µM on EPS on media lactate increase was weakly significant (p=0.0105). In moles, the mean change in media lactate over 24 hours induced by EPS with and without 25µM 2DG was +26.7mM and +30.6mM lactate respectively.


Media Lactate
EPS only
****
Lactate concentration (mM)
30
20
10
0
0
0.5
4
8
24
Time (hours)


Media Lactate
25uM+EPS
****
Lactate concentration (mM)
25
20
15
10
5
0
0
0.5
4
8
24
Time (hours)

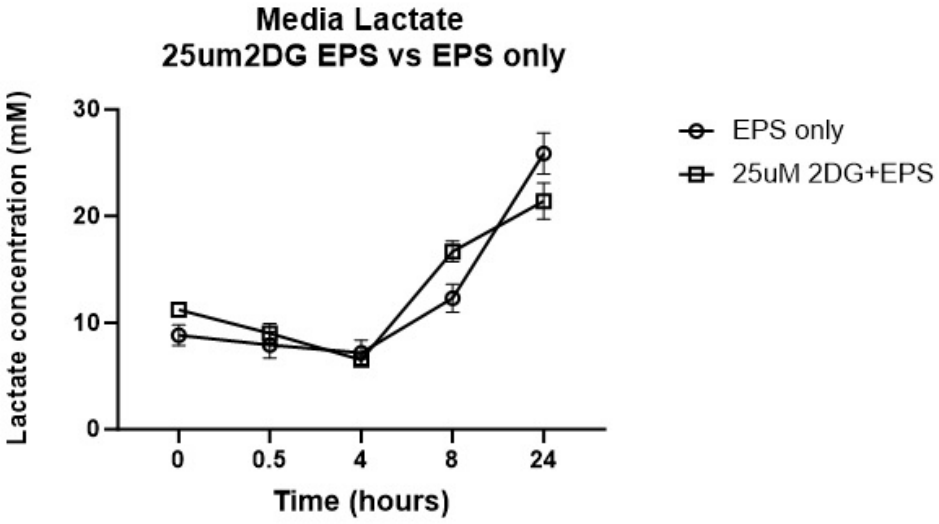

Media Lactate
25um2DG EPS vs EPS only
Lactate concentration (mM)
30
20
10
0
0
0.5
4
8
24
Time (hours)
EPS only
25uM 2DG+EPS

Figure 2.6: Change in media lactate during EPS with and without the addition of 25µM 2DG. Error bars represent standard error of the mean. For both data sets N=6.

2.3.5 EPS induced changes in MPS and MPB

Western blot analysis of cells treated with 25µM 2DG showed changes MTOR (S2448), 4EBP1 (T37/46) and P70S6K (T389) phosphorylation but not GAPDH (**Figure 2.8**), which remained steady over time.

Phospho-MTOR S2448(**Figure 2.7**) showed a significant increase from baseline values at 8 hours (p= 0.0343) and returning to baseline values by 24 hours of stimulation. Similarly, 4EBP1 T37/46 phosphorylation increased sharply to 2-fold baseline by 0.5 hours, but by 4 hours had return to levels that were higher than baseline, but not

Figure 2.7: Change in phosphorylation of mTORC1 relative to the 0-hour control. Note the apparent increase in the 24-hour control. Error bars represent standard error of the mean. N=6

significantly so, where they remained until the end of the experiment. Unlike p-4EBbP1 (**Figure 2.10)** and p-P70SK (**Figure 2.9**), the 24-hour control for p-MTOR S2448 showed significant increase from baseline values (p= 0.0225).

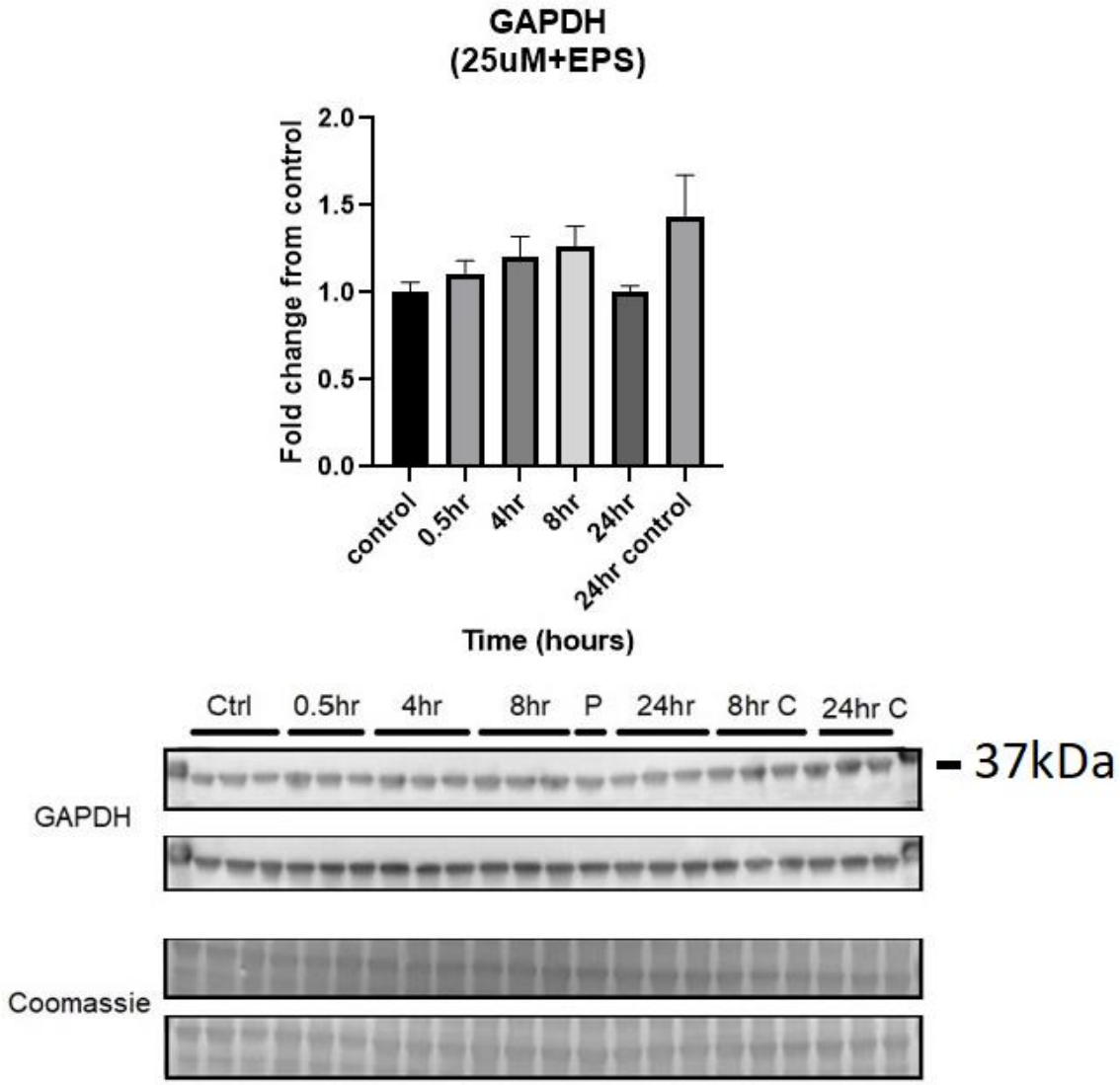


Figure 2.8: Change in phosphorylation of GAPDH in response to 25µM 2DG in combination with EPS. No significant change was observed. Error bars represent standard error of the mean. N=6

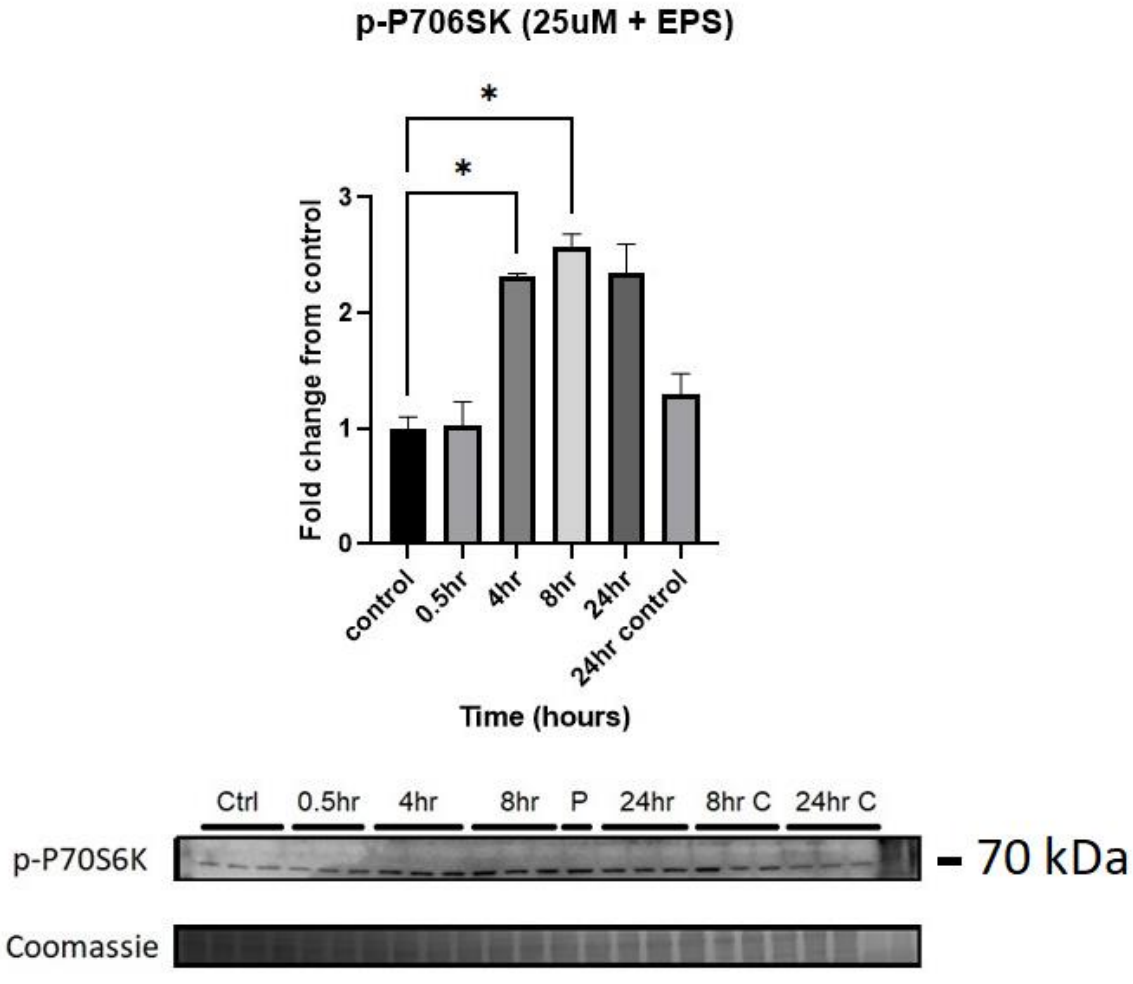


Figure 2.9: Change in phosphorylation of P70S6K in response to EPS with 25 µM 2DG**.** Error bars represent the standard error of the mean. each timepoint consists of 3 repeats (N=3)

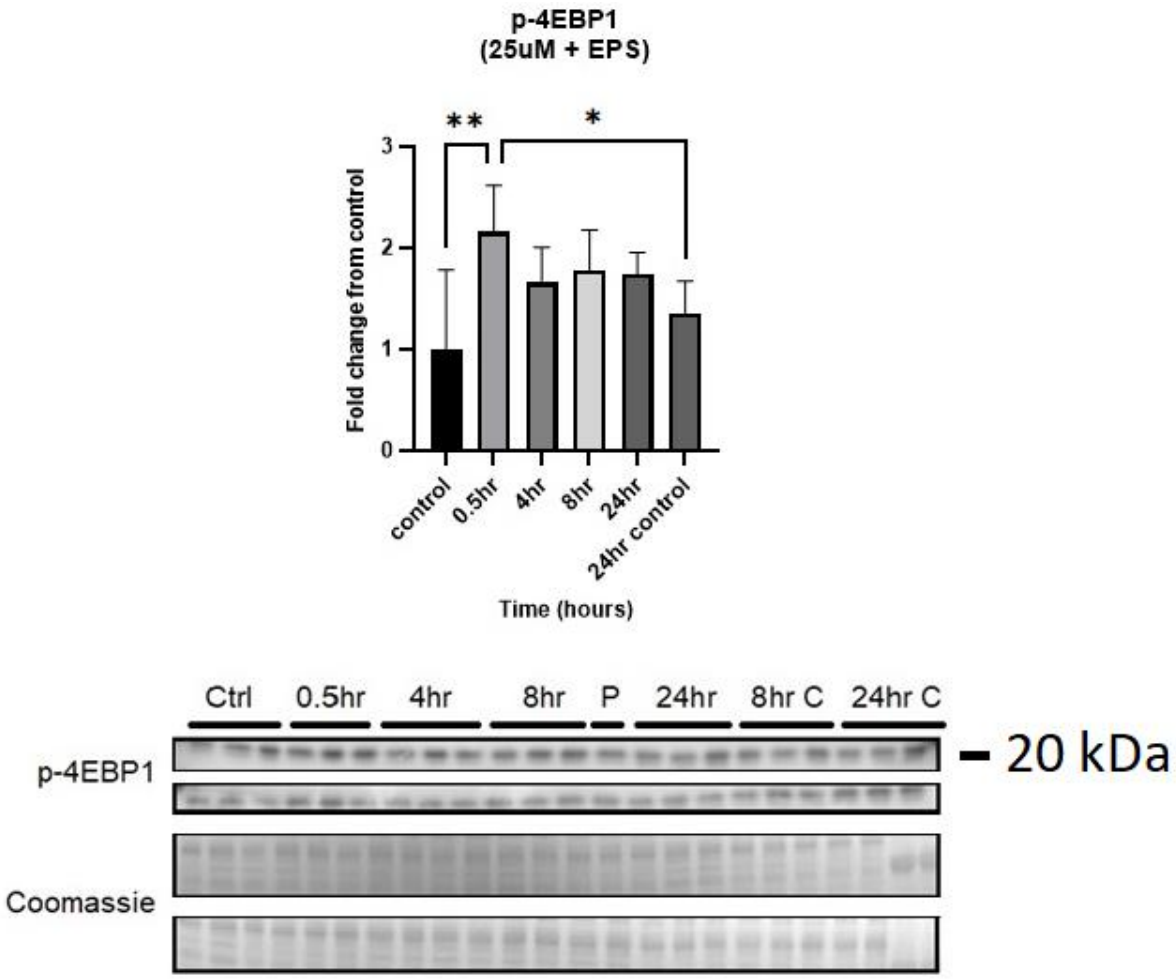


Figure 2.10: Change in phosphorylation of 4EBP1 in response to EPS with 25 µM 2DG**.** Error bars represent the standard error of the mean. each timepoint consists of 3 repeats (N=6)

The Phosphorylation of P70S6K increased 2.1-fold after 4 hours of EPS and increased marginally above this after 8 hours of EPS. The significance of these two increases over baseline were p= 0.0131 and 0.0263, respectively. The level of elevation in phosphorylation was similar at 24 hours to what was seen at 4 hours, but was not significantly different from the control value, presumably due to greater variance among the sample values.

2.4 Discussion

2.4.1 Cell culture conditions and variation in glucose metabolism

It has previously been shown that EPS can induce exercise like effects in cultured skeletal muscle (Nikolic et al, 2012) and that glucose analogues can be used to detect glucose uptake in vitro (Chen et al, 2015). We found that 25µM 2-DG but not 200 µM was suitable for tracing glucose over 24 hours as determined by linearity of uptake and lack of significant change in lactate output. Changes in glucose metabolism including reduction of glucose uptake rate caused by 200 µM D-DG made the higher dose unsuitable for tracing. Additionally, we found that EPS produced the expected changes in c2c12 cells, causing an increase in glucose uptake, lactate output as well as inducing time appropriate alterations in anabolic signalling through mTOR, P70 and 4EBP1 phosphorylation at relevant phosphorylation sites (Ser 2448, Thr 389 and Thr 37/46 respectively).

We determined that 200µM of 2-DG was too high a dose to trace glucose over 24 hours without disruption of glucose metabolism, as shown by the changes to glucose uptake in figure

2.3. Additionally, the lactate output of cells provided with 200µM of 2-DG was significantly higher than equivalent control cells (Figure 2.4). As a result, we revised the dosage down to 25 µM. This resulted in linear uptake of glucose and no change in lactate output, something that had been shown with other glucose analogues before but never with 2-DG to our knowledge (Chien et al, 2020).

We determined that in our hands EPS could be used to induce an exercise like response in glucose metabolism as seen by the increase in both glucose uptake (figure 2.3) and lactate output (Figure 2.6) that was indicative of increased glycolysis. Whilst this use of EPS is not novel, it was required for establishment of the disuse model in later chapters.

We also saw changes in protein synthesis related signalling occur during the EPS protocol that were indicative of changes in cellular function towards increased protein synthesis, with increases in mTOR and P70 phosphorylation as well as decrease in 4EBP1 phosphorylation.

In order to half the volume of samples and number of western blots required we elected to quantify protein phosphorylation against total cell protein as determined by Coomassie stain, rather than against total (Phosphorylated and unphosphorylated) target protein. Whilst this is an accepted technique (Eaton et al, 2013), it does have less sensitivity than comparison between total phosphorylated target and total target, which can detect more granular changes in the ratio of phosphorylation (Tarum et al, 2017)

2.4.2 Investigating muscle function in vitro

A number of papers have compared cell lines for suitability for investigation of treatments intended to manipulate glucose uptake and investigate insulin sensitivity (Wong et al, 2020; Elkalaf et al, 2013; Luo et al, 2019). The conclusion drawn is that growth and differentiation conditions have a significant effect on the metabolic state of cells. Whilst this does reflect

whole animal glucose metabolism, it introduces variables to be controlled. Specifically, the relative levels of IGF-1, insulin and glucose as well as glutamine have important roles in the basal response to provision of glucose as well as growth and differentiation characteristics. To control for this, cells were always fed 24 hours before the beginning of an experiment. Physiological glucose concentrations in healthy humans ranges from 3.5-5.5 mM/L (Guemes et al, 2016). This is by far lower than the high glucose media used for the culture of C2C12 myotubes (24-25 mM/L depending on brand). Similarly, resting insulin concentrations in humans are typically around 21µU – equivalent to 167pM/L (Polonsky et al, 1988). The concentration of insulin provided during growth in the form of FBS and during differentiation as horse serum is unknown and will vary by production lot. The amount of insulin provided to cells per well in this study is 50nM. This is an intermediate value compared to those given in other studies (100nM, 10nM, 17.2nM) (Yun et al, 2009; Tortorella and Pilch, 2002; Huard et al, 2007; Liu et al, 2020; Sanvee et al, 2019) which have used both concentrations for incubation periods between 10 minutes and 16 hours. Our selection of 50nM insulin produced a 1.7-fold increase in lactate production after 24 hours exposure, similar to the value of 1.65-fold increase reported by Hirabara et al (2009) during 2-hour incubation with 7nM insulin. Overall, where possible we picked conditions that would create stable baseline which, though performed in a mouse cell line, recreates conditions relevant to human physiology.

2.4.3 2-Deoxyglucose as a tracer

Suginohara et al (2021) found that 500mg/Kg 2DG inhibited glycolysis in rats.

In our cell culture experiments, in terms of glucose uptake, 200µM 2DG clearly impeded further glucose uptake as a result of the non-metabolizable nature of 2DG6P. Use of radiolabelled 2DG as a glucose tracer has been successfully accomplished by our work group (Chien et al, 2020) and by others (Turinsky et al, 1996; Mooradian and Haas, 2021), however

the use of unlabelled 2-DG and measurement by mass spectrometry as performed in the current work is advantageous as it eliminates the need for radioactive materials and does not rely on measurement of fluorescence, which can be impeded by improper selection of laboratory plastics. Our method also has the additional benefit of being relatively high throughput due to automation of the mass spectrometry portion of measurement and samples are stable at room temperature for several weeks after derivatization, so long as they remain sealed, which gives ample opportunity to revisit samples of interest, something which cannot be done with scintillation counting and fluorescence quantification methods.

2.4.4 EPS as an exercise model

Having verified contraction was occurring by bright field microscopy, we were interested in determining what effect the chosen pulse train settings had on anabolic signalling, as demonstrating impairment of this signalling would be critical for establishing a model of disuse.

The western blot of 4EBP1, P70s6K and mTOR phosphorylation in cells subjected to EPS with 25µM 2DG showed mixed results in terms of evidence for or against alteration of MPB/MPS as a result of 2DG induced glycolysis inhibition. Typically, factors which are pro-MPS are downregulated during exercise to conserve energy for contraction (Kumar et al, 2009). In the rest period after exercise, pro-synthetic pathways are seen to become upregulated, with differences between endurance and resistance exercise (Mitchell et al, 2012; Ahtiainen et al, 2015). 4EBP1 and P70S6K are both downstream effectors of mTORC1, that are also subject to mTORC independent regulation. Suginohara et al (2021) found that high intensity EPS increased P70S6K phosphorylation and decreased 4EBP1 phosphorylation, as well as evidence of mTORC1 activation in the form of raptor phosphorylation increase. Our own findings show

an increase of mTORC1 phosphorylation at the 8-hour timepoint as well as increases in the phosphorylation of the downstream effectors P70S6K and 4EBP1 after 4 and 0.5 hours respectively.

One possible explanation for the difference in findings could be that the response of whole animals to EPS incorporates signalling from multiple tissue types, especially adipose tissue, which is not present in a cell model. A more likely source of difference, Suginohara's protocol was formulated to mimic high intensity resistance exercise (RE) (30V, 100Hz, ten 3-second contractions with 7 second interval) whilst our own protocol was more similar to light endurance exercise (EE) (11.5V, 1Hz, 2ms pulse duration every second). It is known that many changes previously associated with exercise, respond more strongly or exclusively to RE rather than EE or vice versa. Vissing et al (2013) found that when compared to untrained, unexercised individuals, athletes participating in EE did not show significant upregulation of MTORC1, whilst those participating in RE did. This scenario has previously been demonstrated by Atherton et al (2005) using stimulation settings of 50V, 10Hz with 10ms contraction every tenth of a second for 3 hours. This still does not explain the rise in phosphorylation of mTORC1 at the 8-hour timepoint. Without blotting for total mTOR it is not possible to rule out an increase in total mTOR, of which a proportional amount would be phosphorylated as part of the basal regulation of MTOR.

This explanation would also go some way to explaining the changes in 4EBP1 and P70S6K in that before the 8-hour mark, both would be under the influence of mTOR independent regulators, such as MAPK p38 (Qin et al, 2016). The combination of confirmed contraction and lack of suppression of muscle protein synthesis enabling pathways suggests that although the EPS setting used evoked contraction, it was of a type not representative of that seen during skeletal muscle exercise, where suppression of both P70S6K and 4EBP1 phosphorylation can be

seen even in the absence of changes to mTORC1 phosphorylation (Atherton et al, 2005). However, there is precedent for the observed changes as noted by Ogasawara et al, (2014), who draws attention to the finding that protracted low frequency EPS can induce MPB/ MPS changes similar to endurance exercise does in vivo and for this reason it is often preferable to use animal models when simulating EE. This is similar to a study of cardiac muscle during light and heavy endurance exercise by Liao et al (2015). In this study hypertrophic signalling- including that of mTORC1 was upregulated during light EE of animals but not heavy EE. This demonstrates that hypertrophic signalling can exist alongside low frequency and intensity contraction (such as basal heart rate and low frequency EPS). Indeed, C2C12 cells have been shown to adopt some cardiac muscle-like responses when placed in an environment similar to that of the heart- primarily continuous low frequency stimulation (present here) and cardiac muscle conditioned media (not present here) (Zebedin et al, 2007). The results that we have obtained fall within the scope of explanation using existing literature and as such we would interpret them as a response to EPS rather than an artifact or erroneous finding.

2.5 Conclusion

In summary, our aim of measuring of glucose uptake in unstimulated cells was successfully achieved, though glucose metabolism was disturbed in the process. 200 µM 2DG caused increases in lactate concentration in the absence of EPS and resulted in non-linear glucose uptake. Revisions were made- decreasing provided 2DG from 200µM to 25µM. This reduced 2-DG dose was found to have minimal effects on glucose uptake and lactate output, as determined by linear glucose uptake over the measure period of disturbance of glucose metabolism over 24 hours. The finding that contraction can be successfully induced in cells,

with accompanying changes to anabolic signalling that fall within expected ranges for the C2C12 cell line is encouraging. Increases in media lactate as well as phosphorylation of mTORC1, P70S6k and 4EBP1 are also positive signs of the efficacy of an EPS protocol of 24 hours, 11.5V, 2ms, 1Hz on inducing measurable effects, though they also highlight that there are differences between the expected outcomes from low frequency EPS and the actual outcomes, the wider literature shows that with some adjustment in the assumptions around what the current EPS protocol achieves, EPS can still be used to mimic contraction in muscle, so long as these discrepancies are kept in mind. These findings make the C2C12 cell line a suitable platform to base a model of muscle disuse.

Chapter 3: Effect of varying frequency of intermittent EPS on glucose uptake and related signalling.

3.0 Introduction

3.0.1 Role of muscle in glucose uptake

Skeletal muscle is essential to the normal regulation of glucose throughout the body. At rest the majority of the energy required by the body is acquired from fat oxidation (Melzer, 2011). In metabolically normal people, this changes during exercise and as exercise intensity approaches maximum, so too does the proportion of whole-body energy that is derived from glucose oxidation (Rose and Richter, 2005), with skeletal muscle accounting for up to 85% of glucose disposal (Wendt *et al*, 2021). Correct regulation of glucose throughout the body is important because high concentrations of glucose in blood can lead to inappropriate glycosylation throughout the body, which has a number of deleterious effects including increased oxidative stress, abnormal vasculature and inflammation in tissues (Negre-Salvayre *et al*, 2009).

Skeletal muscle imports glucose from the bloodstream primarily through the glucose transporter GLUT4. This channel works via facilitated diffusion across a concentration gradient. GLUT4 can be separated into two pools, which are recruited independently from one another. The insulin sensitive pool is recruited in response to the binding of insulin at the insulin receptor, which -through IRS-1- incites the activation of PI3K-Akt-AS160 pathway, resulting recruitment of GLUT4 to the plasma membrane. The contraction sensitive pool responds to exercise rather than insulin. Despite being referred to as “contraction sensitive”, it should be noted that it is mechanical transduction of force that activates this pathway, as passive stretching of muscle is sufficient to activate the “contraction sensitive” GLUT4 recruitment pathway (Richter, 2021). Though it has never been decisively shown, it is thought that perhaps

the activity of the GLUT4 transporter can be increased by (and during) exercise, as the doubling of GLUT4 in the membrane is associated with a glucose uptake increase of up to 100 times.

3.0.2 Exercise Dosing and Glucose uptake

Changes in the uptake and fate of glucose depending on the intensity of exercise have been speculated upon for some time, given that blood lactate concentration only increases with exercise above a certain intensity threshold (Cooper *et al*, 1989) and it has been shown that high intensity exercise can spike blood glucose through stress hormone production, causing hepatic release of glucose (glycogenolysis) (Trefts *et al*, 2015).

As well as changing intensity of exercise to increase glucose uptake, the duration of exercise will naturally alter glucose uptake, with a longer exercise session requiring more generation of ATP – and therefore more uptake of glucose- than a shorter session of equal intensity (Soo *et al*, 2023). This has led to the belief that it is the work done, rather than intensity or duration alone, that is the most important determinant of the efficacy of exercise as an intervention for elevated blood glucose (Colberg *et al*, 2013), though this concept is still disputed (Fillmore *et al*, 2010).

3.0.3 Cell culture models for studying skeletal muscle contraction

Cell culture models for exercise have been a subject of interest in recent years, with multiple groups showing successful contraction of cultured myotubes with origins in humans (Guo *et al*, 2014) and rodents (Abdelmoez *et al*, 2020). The main advantage of a cell culture model over animal models or isolated muscle groups is the ability to identify effects that have their origin solely in muscle, rather than as a response associated with factors secreted from other tissues. It allows us to deepen our understanding of the function of muscle itself. Whilst these successful contraction models have shown exercise like responses to contraction in terms of myokine release (Furuichi *et al*, 2018), capturing the exact changes induced by exercise on a

timescale that we would recognize as physiologically relevant has been difficult, likely owing to differences in contraction parameters between work groups (Carter and Solomon, 2018), differences in the timepoints of measurements, as well as differences inherent to donors for human primary cells and differences between cell lines (Abdelmoez *et al*, 2020). What has been established in C2C12 is that electrical current can be used to induce contractions in myotubes, with recruitment of GLUT4 being enhanced (Nedachi *et al*, 2008), glucose uptake and lactate production enhanced (Nikolic *et al*, 2012) and changes in the transcriptome that are similar to the effects of exercise in mouse models of exercise (Lee *et al*, 2022). Though there have been some studies that look at the effect of repeated bouts of exercise, methods have been disparate, with Burch *et al* (2010) showing one 90 minute "bout" of EPS per day for 4 days upregulated PGC-1α in C2C12 myotubes in line with a normal exercise response. Another study by Melouane *et al* (2019) used 8 hours of low frequency EPS on day one, followed by 16 hours of high frequency EPS on day 2. They showed upregulation in PGC-1α and osteonectin in response to this. Most investigations involving alternating EPS conditions focus on changing the settings of the pulse train, rather than introducing a gap into the application of the pulse train (Brown *et al*, 2015). Because of this, there is almost no data on the effect of rest periods of multiple hours on changes in protein synthesis related signalling or glucose uptake, when it seems that this would be a relevant parameter when considering extrapolating results obtained *in vitro* to future work in animals and participants.

3.0.4 Aims and Hypothesis

We aimed to determine the point between continuous and no EPS at which there were diminishing returns on glucose uptake or protein synthesis measurements. We hypothesized that, as linear uptake had been established over 24 hours of continuous EPS, the glucose uptake response would be directly proportional to the time spent contracting. We expected that changes in pro-synthesis signalling may reach maximum intensity or saturation after only a

few hours of EPS, indicating that a certain number of bouts of EPS per day would cause a plateau in the signalling of phospho-mTOR, phospho-P70, Phospho-4EBP1 and phospho-PKB.

3.1 Methods

3.1.1 Cell culture

Cell culture of C2C12 cells was performed as described in chapter 2. Cells used in the intermittent EPS experiments were from 2 separate seedings, resulting in the 1EX experiments using cells of passage 9, whilst 2EX and 3EX were of passage 10. Both seeding vials originated from the same stock. For the purposes of this chapter, n=3 refers to 3 wells of a plate.

3.1.2 Intermittent EPS protocol

Rather than use the continuous contraction settings described in chapter 2, intermittent EPS was performed with contractions varying in either frequency or duration per 24 hours. Contraction patterns are labelled starting with the number of hours that each block of contractions will last and ending with the number of hours elapsed before the next set of contractions. **Figure 3.1** shows the complete list of contractions. Frequencies were chosen based on their ability to be paired with another duration that amounted to the same total number of hours spent contracting per 24 hours, but at different intervals.

Pulse train settings were maintained at 11.5V, 2ms, 1Hz as described in chapter 2, with a 1-hour pre-stimulation period to induce contractile sarcomere formation.

3.1.3 Lactate assay

Measurement of media lactate concentration was performed as described in chapter 2

3.1.4 Glycogen assay

Measurement of cell glycogen was performed as described in chapter 2

3.1.5 Glucose uptake

Measurement of glucose uptake as internal 2DG6P was performed as described in chapter 2, with slight modification. Rather than performing 1 injection of 6 separate samples, 3 samples were used for each timepoint, injected twice. This modification was made to reduce mass spectrometer running time and cell culture requirements.

3.1.6 Measurement of signalling

Anabolic signalling was measured by western blotting as described in chapter 2. Additionally, targets relating to glucose uptake and metabolism were blotted- GLUT4, GLUT1 and PKB (Akt).

3.1.7 Data analysis

Data analysis was performed as described in chapter 2.

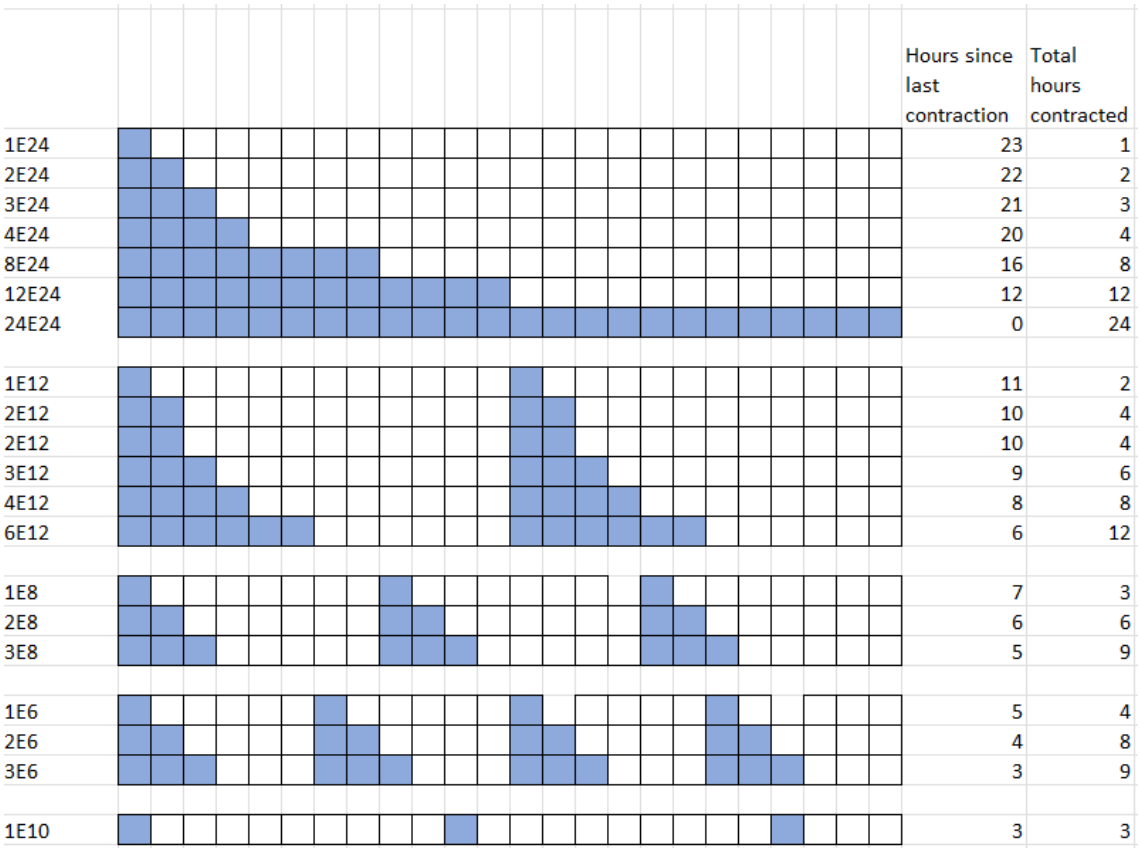


Figure 3.1: Diagrammatic representation of intermittent EPS protocols. One blue box indicates one hour of continuous EPS. A white box indicates an hour in which no EPS is applied. Cells were then harvested at the end of the 24th hour.

3.2 Results

3.2.1 Changes in lactate in response to intermittent EPS protocols

After an hour of pre-stimulatory EPS, cells were paced for 24 hours on one of several protocols which would consist of a number of hours of EPS, followed by a number of hours resting. At the end of the 24 hours, cells were harvested. It was found that none of the EPS protocols applied here (**Figure 3.2**) induced a significant increase above the media lactate level recorded for unstimulated cells. The greatest increase in the 1EX group was from 8.8 +/- 0.9mmol to 11.3 +/-

0.6 mmol. For the 2EX group the greatest decrease in media lactate came from 2E24 (8.8 +/- 0.8mmol to 5.9 +/- 0.3 mmol). The greatest increase in the 2EX group was minimal, increasing to 9.3 +/- 1.0mmol. The 3EX group showed the most change from the unstimulated state, with 3E24 increasing to 17.4 +/- 3.8mmol. The only significant difference was between the intermittent EPS protocols and a 24-hour continuous stimulation protocol ($p<0.0001$).

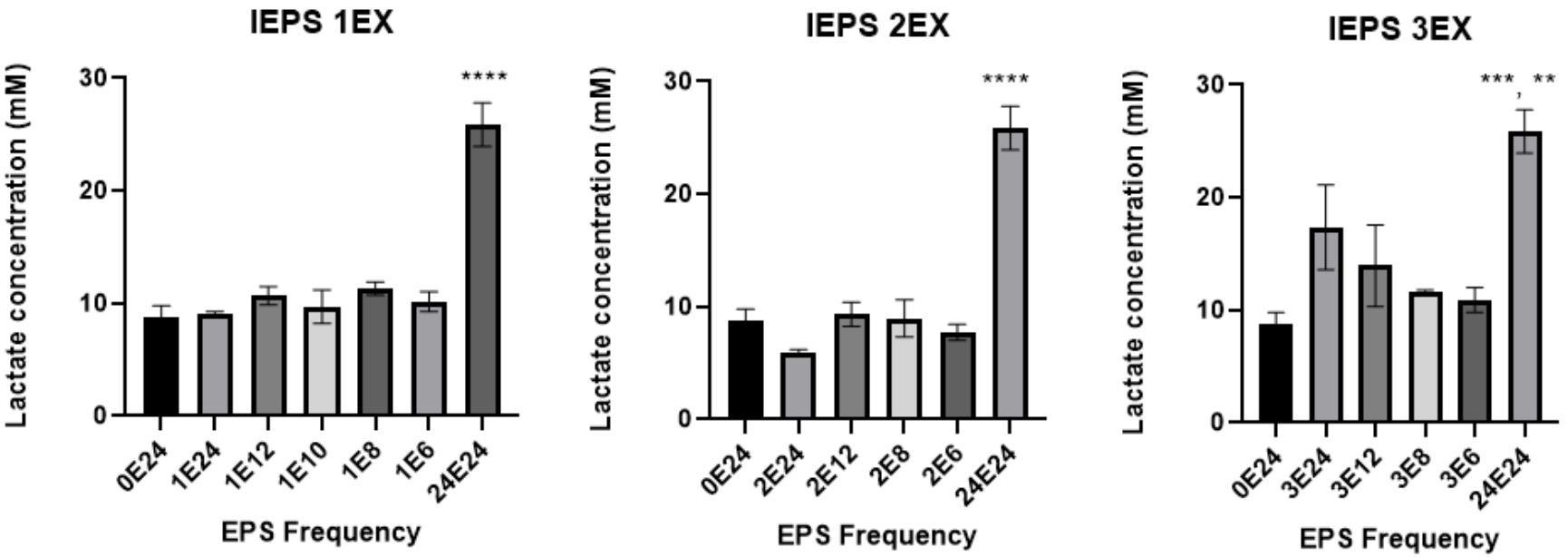


Figure 3.2: C2C12 cells were subjected to EPS intermittently for 24 hours. For example, 1E12 indicates 1 hour of contraction every 12 hours, indicating 2 hours of contraction before harvesting at 24 hours. From left to right, graphs show the media lactate concentration of C2C12 cells provided increasing durations of EPS at the same frequency intervals. **** indicates that the 24hr stimulation protocol was significantly different from all other protocols ($P<0.0001$). *** indicates difference from 3E8 ($P<0.005$). ** indicates difference from 3E6. Values are mean ± SEM (n=3).

3.2.2 Changes in glycogen in response to intermittent EPS protocols

As with lactate, changes in glycogen concentration when compared to the unstimulated state did not reach significance for any of the protocols. This is shown in **Figure 3.3**. There was no difference recorded between any of the experimental protocols, though there was one significant result recorded. When comparing the 1E6 protocol with 24-hour continuous EPS, there was a weakly significant difference (p=0.015) owing to the lower-than-average glycogen content of cells which had received this protocol. This was a decrease of 2.4mmol (from 4.3 +/- 0.5mmol to 1.9 +/- 0.5mmol. Glycogen remained steady for most of the 2EX group, with 2E24 producing the largest decrease (down to 2.8 +/-0.1 mmol). Conversely, the 3EX group's glycogen either remained the same or increased. Though 3E12 produced a large increase to 6.8mmol, the margin of error was large (+/- 2.2mmol).

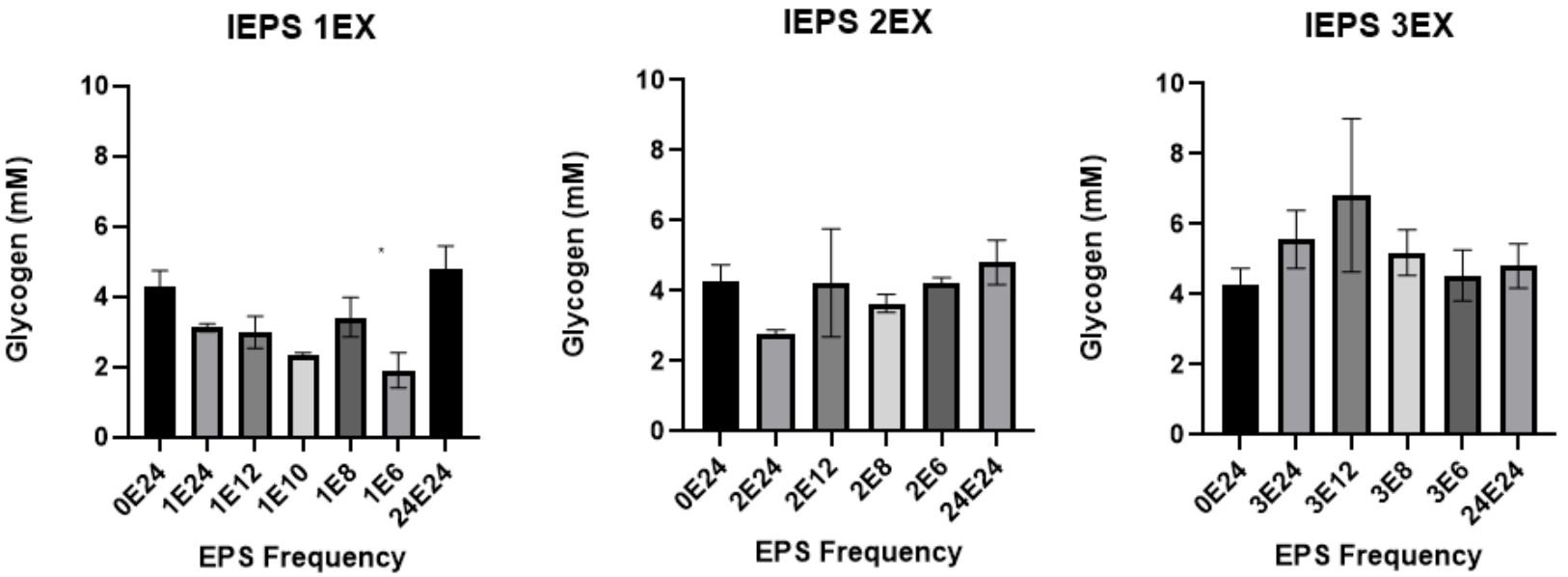


Figure 3.3: C2C12 cells were subjected to EPS intermittently for 24 hours. For example, 1E12 indicates 1 hour of contraction every 12 hours, indicating 2 hours of contraction before harvesting at 24 hours. From left to right, graphs show the glycogen concentration of C2C12 cells provided increasing durations of EPS at the same frequency intervals. * Indicates that 1E6 is significantly different from 24E24 ($p=0.015$). Values are means +/- SEM (n=3).

3.2.3 Changes in Glucose uptake in response to intermittent EPS protocols

Glucose uptake was in line with lactate and glycogen measurements, with no significant change from unstimulated values. However, as a weak visual trend, particularly when looking at 2EX and 3EX protocols (**figure 3.4**), increasing duration of stimulation increased uptake, whilst greater frequency lowered uptake, producing the stepped profile from left to right within each graph. Again, the only protocols significantly different from the others was 24 hours continuous EPS ($P<0.0001$).

Whilst there was no difference in glucose uptake between protocols which had matched work done (**Figure 3.5B**), there was also no difference in glucose uptake between the protocols which differed in work done by as many as 12 times (**Figure 3.5A**)

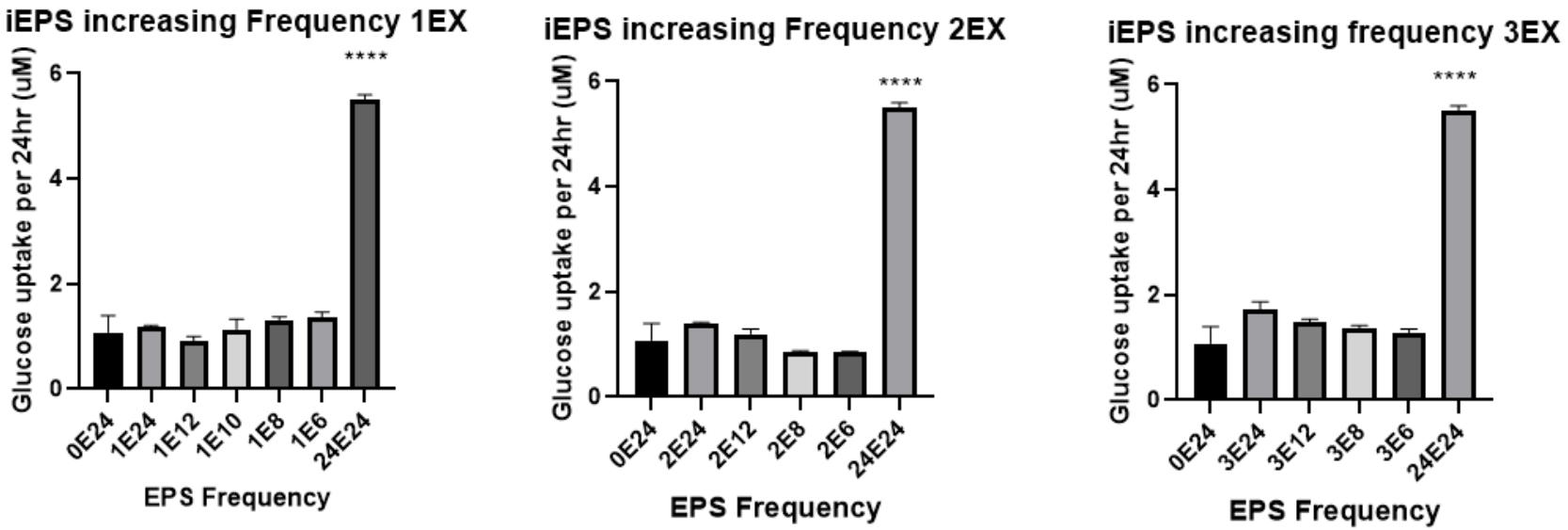


Figure 3.4: C2C12 cells were subjected to EPS intermittently for 24 hours. For example, 1E12 indicates 1 hour of contraction every 12 hours, indicating 2 hours of contraction before harvesting at 24 hours. From left to right, graphs show the glucose uptake of C2C12 cells after 24hr, provided increasing durations of EPS at the same frequency intervals. The only significant difference was 24E24, which differed from all other protocols ($P<0.0001$). Values are means +/- SEM (n=3).

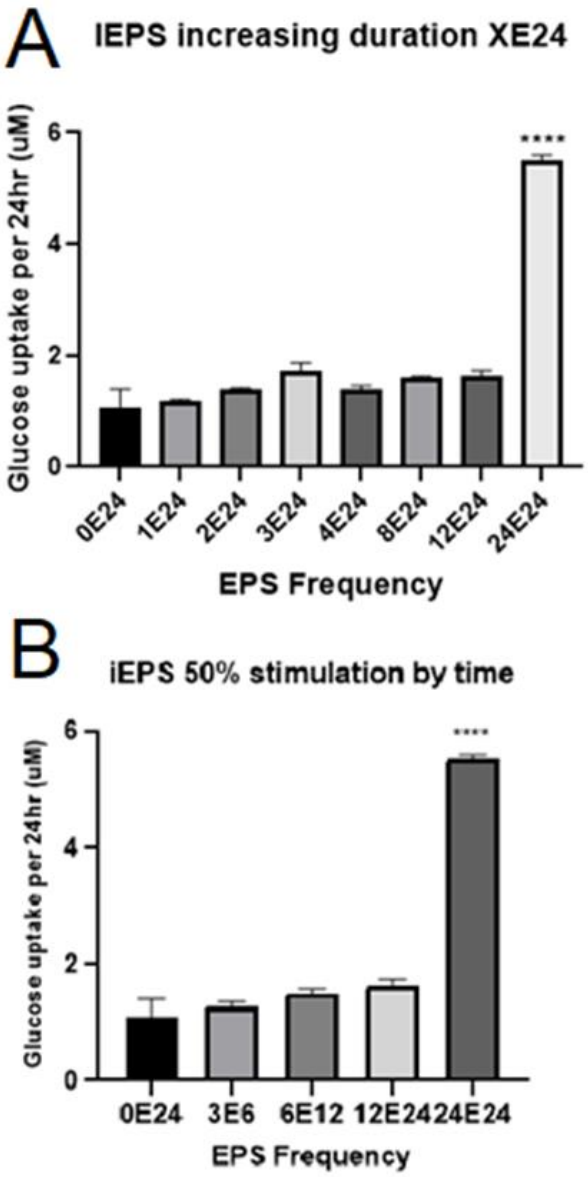


Figure 3.5: (A) Increasing duration of EPS up to 12 hours of continuous EPS [per 24 hours] did not significantly increase glucose uptake when compared to incrementally shorter durations. (B) Comparison of several different EPS frequency protocols which each equate to 50% of a 24 -hour period contracting. A small non-significant increase in uptake with increasing duration (and therefore decreasing number of bouts) was observed. **** Indicates 24E24 differs from all other protocols significantly ($P<0.0001$). Values are means +/- SEM (n=3).

3.2.4 Signalling changes in response to intermittent EPS

Cells scraped in homogenization buffer were prepared as described and run on Bio-Rad gels. Gels were normalized by the presence of the loading control, which was also the 0E24 -or unstimulated- sample.

At the lowest EPS duration- 1hr (**Figure 3.6)**- anabolic signalling appeared to decrease with increasing frequency. At the greatest frequency of 1hour EPS per 6 hours, P70 phosphorylation fell to 35% of the unstimulated value. This was in line with the change in mTOR which was 69% of the unstimulated value. 4EBP1 phosphorylation for the 1EX series showed a non-significant increase when frequency increased from 1E24 to 1E12 but increasing frequency further beyond this resulted in phosphorylation which was only 70% of the unstimulated value.

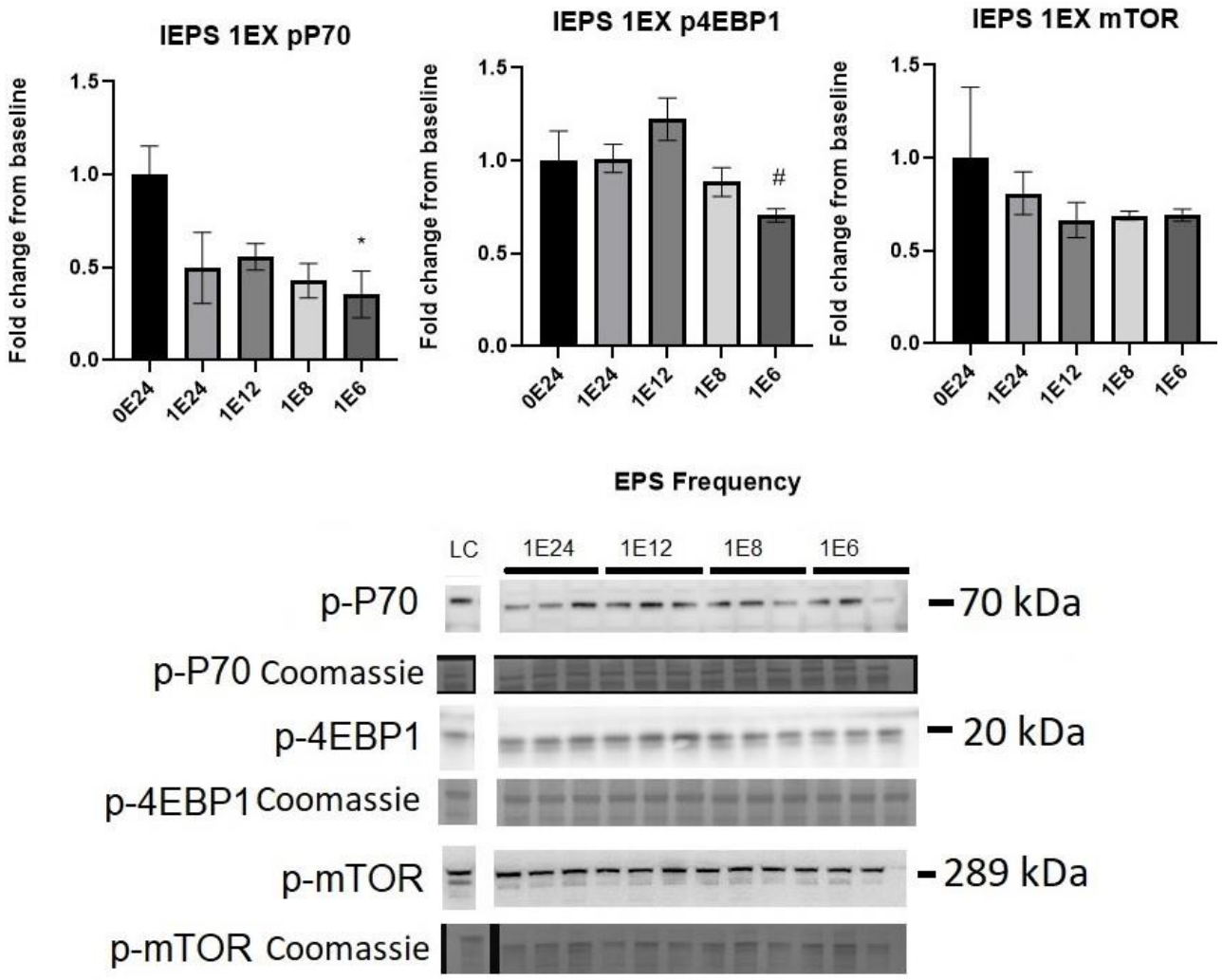


Figure 3.6: quantification of the western blotting of phosphorylated forms of anabolic signaling proteins P70S6K, 4EBP1 and their upstream controller mTOR1 in C2C12 cells. Response to EPS frequencies of one every 24 hours, one hour every 12 hours, every 8 hours and every 6 hours is shown. * Indicates significant difference ($p<0.05$) from 0E24. # Indicates significant difference ($p<0.05$) from 1E12. N=3.

For the intermediate duration of EPS stimulation, in which "bouts" lasted for 2 hours, the patterns seen in changes were different from the 1EX series. P70 phosphorylation increased with increasing frequency of bouts, up until 2E12, after which increasing frequency progressively decreased phosphorylation. The 2E6 frequency significantly ($p=0.0458$) reduced phosphorylation from the unstimulated state by one quarter. The changes in P70 were weakly reflected in mTOR1 phosphorylation, but none of the changes were significant and values never decreased from baseline more than 5%. 4EBP1 phosphorylation showed a steep decline with the introduction of EPS, with increase thereafter with increasing EPS frequency, with 2E6 peaking at 31% greater than 0E24 values ($p=0.0022$) (**Figure 3.7**).

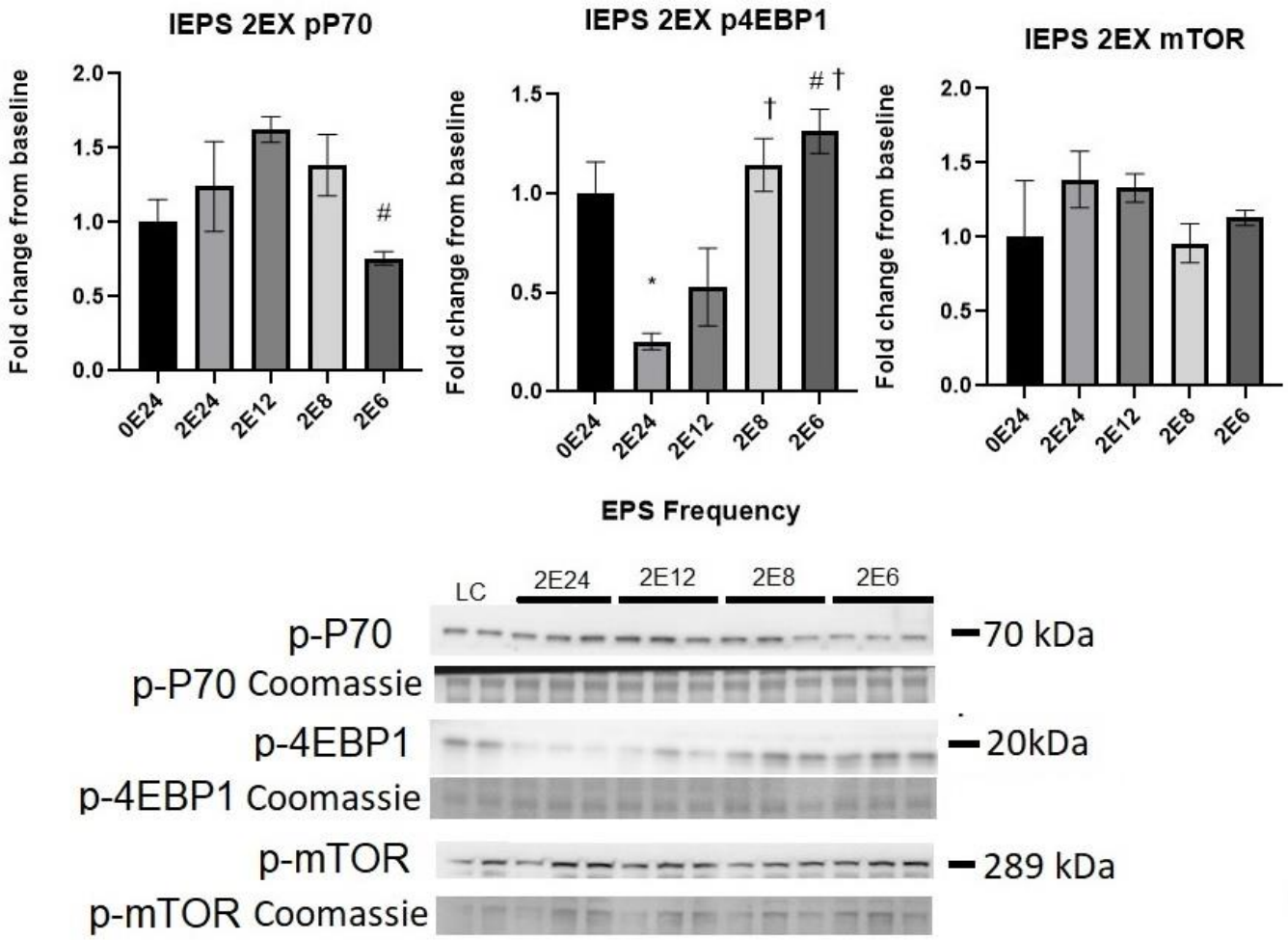


Figure 3.7: quantification of the western blotting of phosphorylated forms of anabolic signaling proteins P70S6K, 4EBP1 and their upstream controller mTOR1 in C2C12 cells. Response to EPS frequencies of two hours every 24 hours, two hours every 12 hours, every 8 hours and every 6 hours is shown. * Indicates significant difference from 0E24. # Indicates significant difference from 2E12. † indicates a significant difference from 2E24. Values are means +/- SEM. n=3.

3EX, the longest duration of EPS per bout, produced changes in anabolic signalling more similar to the 1EX group than the 2EX group. P70 phosphorylation was, as a trend reduced when compared to the resting state, with 3E12 and 3E8 significantly reduced (p=0.0202 and 0.0116 respectively). As 3E24 did not produce a change in phosphorylation, there was also a significant difference between both 3E12&3E8 and 3E24 (p= 0.0136 and 0.0095) (**Figure 3.8**). Again, this was not reflected in mTOR1 which showed a small increase in phosphorylation followed by slight incremental decline, none of which reached significance. Changes in 4EBP1 phosphorylation when comparing to the resting state in the 3EX group were measurable only at 3E24 and 3E12, both of which were elevated ~ 80% above resting values (p=0.0262 and 0.0149).

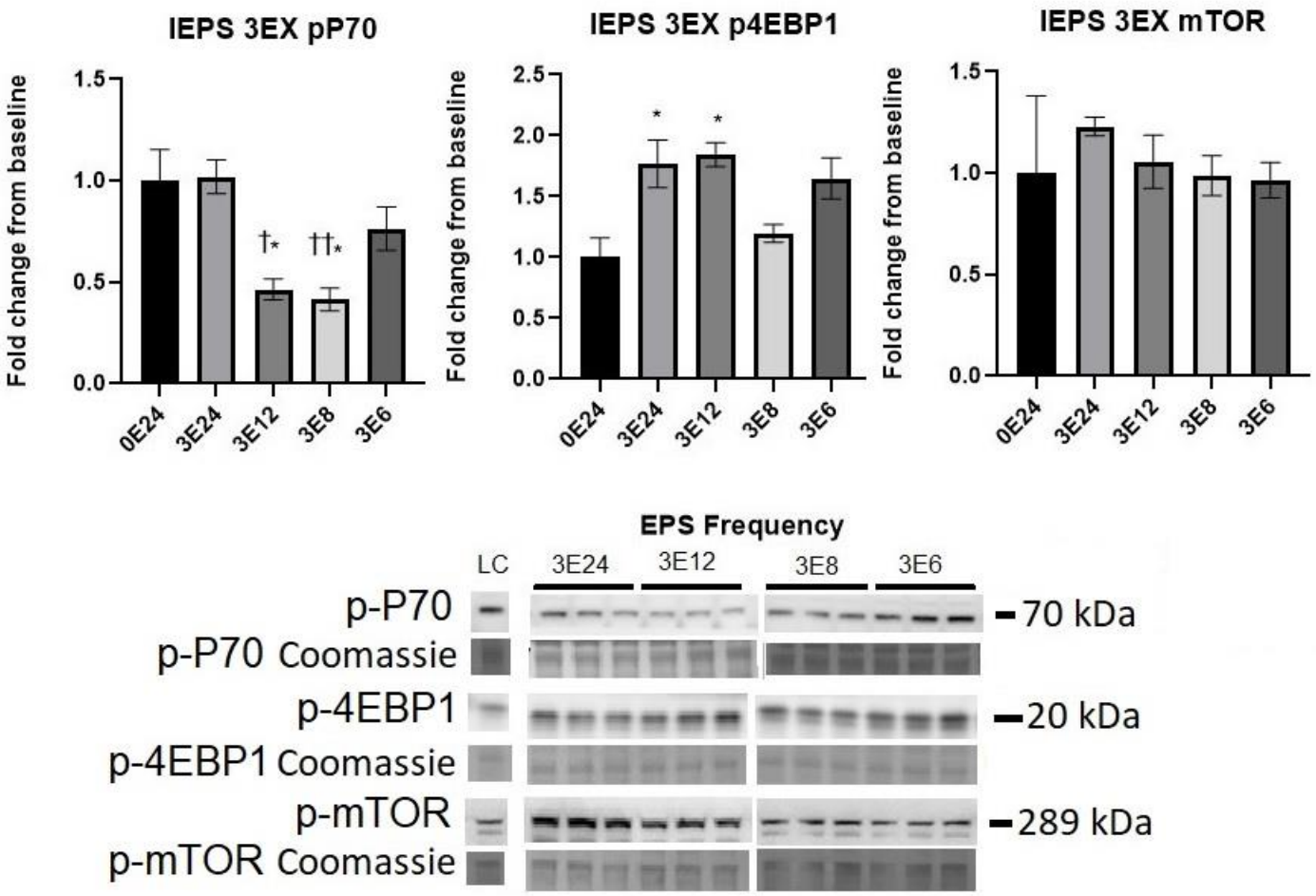


Figure 3.8: quantification of the western blotting of phosphorylated forms of anabolic signaling proteins P70S6K, 4EBP1 and their upstream controller mTOR1 in C2C12 cells. Response to EPS frequencies of three hours every 24 hours, one hour every 12 hours, every 8 hours and every 6 hours is shown * Indicates significant difference from 0E24. † indicates significant difference ($p<0.05$) from 3E24 † † indicates $p<0.005$. Values are means +/- SEM (n=3).

Finally, changes in Akt/PKB phosphorylation were measured in response to the three durations of EPS. All four frequencies decreased PKB phosphorylation to a greater or lesser extent regardless of the duration (**Figure 3.9**). The duration groups 1EX and 2EX showed a trend of decreasing phosphorylation of PKB with increasing frequency. However, 3EX showed increasing phosphorylation of PKB- from the heavily depressed state induced by any amount of EPS- with increasing frequency. Even so, at the highest frequency and duration combination of 3E6, PKB phosphorylation was decreased by approximately one third when compared to the unstimulated state ($p= 0.0181$).

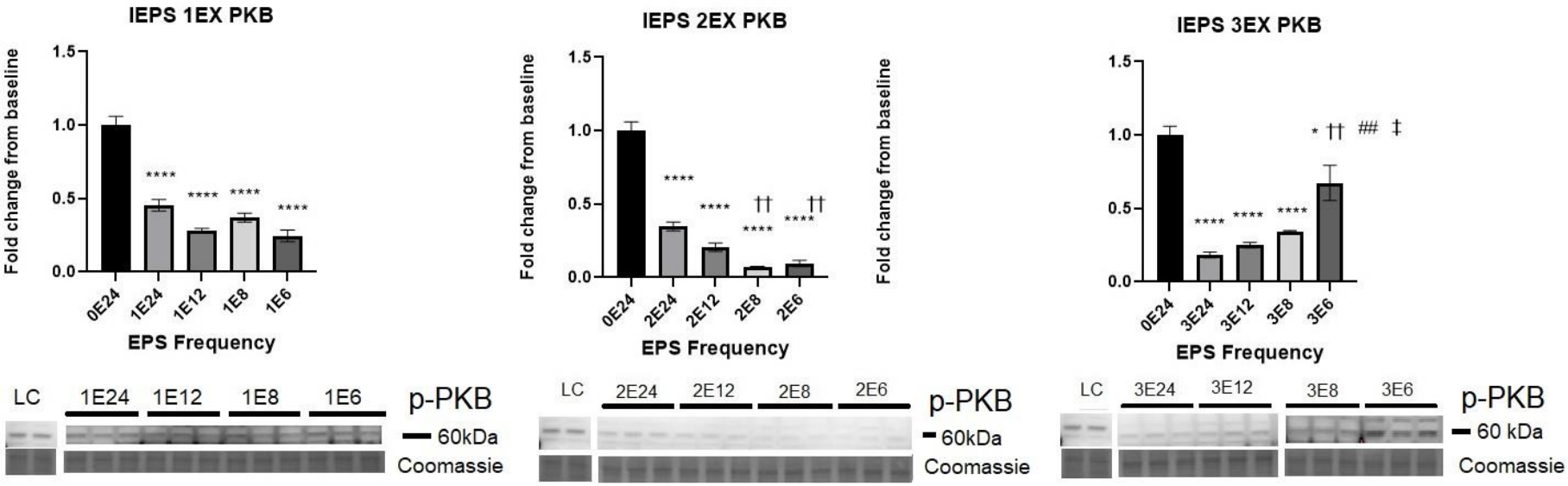


Figure 3.9: quantification of western blotting of PKB-Thr308 phosphorylation. * Indicates significant difference ($p<0.05$) from 0E24, whilst **** indicates $p<0.0001$. † indicates a significant difference ($p<0.05$) whilst † † indicates $P<0.005$ from XE24. # Indicates a significant difference ($p<0.05$) from XE12. ‡ indicates a significant difference ($p<0.05$) from XE8. Values are means +/- SEM. N=3.

3.3 Discussion

The main finding of the experiments listed above is that- within a 24-hour window- intermittent EPS does not appear to significantly increase secretion of lactate into the surrounding media, total size of cellular glycogen store, or the uptake of glucose when compared to unstimulated C2C12 cells. Despite this, changes in anabolic signalling were observed though they did not behave in a consistent manner when considering duration of EPS and duration of the rest period. Decreases in the phosphorylation of PKB1, an important kinase in both the insulin dependent and independent GLUT4 recruitment pathways were consistently observed in response to all intermittent EPS protocols.

3.3.1 The impact of intermittent EPS on glucose uptake

The uptake of glucose by the intermittent EPS protocols chosen did not significantly increase from unstimulated values. Importantly, the differences between protocols were not linearly proportional to the number of hours cells spent contracting. This was reflected by the data obtained by measuring the media lactate concentration.

Media lactate concentration did not change significantly from the unstimulated state in response to intermittent EPS, at least at the 24-hour mark captured here. The greatest change was seen in 3EX- the group with the longest duration of EPS per bout. This change was equivalent to a 1.9-fold increase but was not significant.

Apart from 1E6, none of the intermittent EPS protocols caused a significant change in cellular glycogen compared to continuous EPS. 1E6 caused a slight decrease in cellular glycogen, which is surprising, since it was not harvested rapidly after EPS compared to other patterns of

stimulation. Similarly, the total volume of work performed was relatively low compared to other patterns of EPS which would require more ATP to be performed.

Taken together, the lactate, glycogen and glucose uptake data suggest that the intermittent EPS protocols we applied had no effect- or at least no sustained effect- on glucose uptake or glycolysis.

In the case of glycogen, it is well known that depletion of glycogen during exercise is proportional to the effort required by the exercise as well as to the duration of exercise (Murray and Rosenbloom, 2018). The applied EPS protocol was more similar to aerobic exercise than it was to resistance exercise (Nikolic *et al*, 2018) as shown by comparison to values in mice trained on a running wheel (Son *et al*, 2019). Whilst 1Hz is a low frequency of contraction compared to values obtain during human voluntary movement (20-50Hz (Jones, 1996)) it is necessary to limit the contraction frequency in order to avoid fatigue (Uchimura *et* al, 2021). This could mean that there may have been no decrease at all in glycogen, as was seen in the previous chapter's experiments. Alternatively, owing to the time elapsed between the end of contraction and harvesting, any loss of glycogen may have been masked by the resynthesis of glycogen stores. In humans, resynthesis of glycogen without carbohydrate refeeding occurs at a rate between 1 and 2 mmol/Kg(wet)/hr (Burke *et al*, 2017). Furthermore, it has been shown that in a high glucose media- as was used here- EPS using the same pulse train settings as we used actually increases the size of the glycogen store (Farmawati *et al*, 2013). This matches studies of running in humans, which show increased size of the glycogen store following exercise and feeding due to supercompensation during resynthesis (Murray and Rosenbloom, 2018)

There are several possible explanations for the lack of increase in media lactate in response to most protocols. One is that owing to the work performed being only a fraction of 24-hour

continuous EPS, the lactate produced was also only a fraction of that produced after 24 hours of contraction. In *vivo* the liver removes much of the circulating lactate that remains after exercise (Brooks, 2020). This is not possible in a cell culture environment, so it must be considered that lactate was taken back up inside the cell via monocarboxylate transporters (Juel and Halestrap, 1999) or that it was not produced in the first place. Brooks (2009) reports that in the resting state, half of the lactate taken into the cell is oxidized in healthy humans. Given a resting period of 3 to 23 hours following experimental EPS, it is possible that differences in media lactate were equalized by re-uptake of lactate as part of normal lactate shuttling activity. However, recent findings on the pro-hypertrophy effect of lactic acid on c2c12 myotubes (Zhou *et al*, 2022) suggest that if lactate had been produced as a function of contraction, this would be reflected by changes in P70 mTOR and 4EBP1 phosphorylation changes that would enable hypertrophy, which we did not find.

One possible explanation for the lack of increased glucose uptake in response to intermittent EPS is that the disassembly of sarcomeres during the initial rest phase also disassembled or otherwise isolated the mechanism allowing physical contraction to induce GLUT4 recruitment to the membrane. Fujita *et al* (2010) report that after 2 hours of inactivity, 60% of cells which had previously contained functional sarcomeres no longer did. By 4 hours approximately 75% of cells had lost their sarcomeres. Much of the literature focused on the organization of cytoskeletal elements suggests that dysregulation of structural elements within muscle cells can have an impact not only on the contractile properties but also on the metabolic function. It is known (Zaid *et al*, 2008) that in order for GLUT4 vesicles to correctly translocate to the cell membrane in response to insulin, functional microtubules and filamentous actin is required in both primary cultures and cell lines. Olson *et al (2001)* provide evidence for this in adipose tissue, showing that Nocadozol treatment depolymerized 95% of microtubules, without

influencing the activity of PKB (previously a criticism of this method), resulting in an associated fall in GLUT 4 translocation to less than one fifth the untreated value. In muscle, it is confirmed that loss of the actin filaments arrayed by the plasma membrane results in a 30%- 45% decrease in 2DG uptake in a time dependent manner (Brozinick *et al,* 2004). These papers and others, collated in several reviews (Zaid *et al*, 2008; Batty and Langlais, 2021) highlight the importance of the cytoskeleton in both myotubes and adipocytes for efficient GLUT4 translocation. Recent findings in mice support extrapolation of this requirement to include contraction mediated uptake of glucose as experimental deletion of β-catenin in muscle resulted in a severe reduction in the uptake of glucose elicited by exercise (Masson *et al*, 2021). These findings are in line with previous studies (Salvi *et al*, 2021; Audzeyenka *et al*, 2021) which reaffirm the necessity of cytoskeletal organization for trafficking of GLUT4 bearing vesicles. Given the overlap in function of elements of contraction and insulin stimulated glucose uptake, some (Jensen *et al*, 2014) have suggested that additive effects of AMPK signalling, and contraction/stretch mechanisms produce the total glucose uptake induced by contraction. It could be that for shorter EPS protocols there was insufficient nutrient depletion to greatly activate AMPK, combined with subsequent disassembly of sarcomeric and potentially uptake pathways, which hindered stimulation of glucose uptake.

It is unlikely that 2-DG escaping or export from cells is the cause of the lack of increase in glucose uptake. Firstly, the accumulative mechanism of phosphorylation to 2-DG6P has been described in the literature and also demonstrated in the previous chapter. Additionally, and more importantly, muscle cells do not possess the necessary glucose-6-phosphatase for dephosphorylation and subsequent transport of glucose to the blood stream (Jensen *et al*, 2011) and so can only export lactate. However, 2DG6P cannot be metabolized and so cannot be converted to lactate. These same properties are what allow us to measure the uptake over

time. Though it is not possible in muscle for 2-DG escape or export to occur, it is thought that both export and dephosphorylation of 2-DG6P occurs in neuronal cell lines such as astrocytes (Forsythe *et al*, 1996).

3.3.2 The impact of intermittent EPS on anabolic signalling

The impact of intermittent EPS protocols on anabolic signalling is difficult to discern, based on the lack of obvious patterns in changes to signalling when sorting protocols by contraction session length or contraction session frequency.

PKB showed the most consistent response to altered duration and frequency of EPS, with a consistent decrease in phosphorylation seen for all protocols. This is in line with previous studies on signalling changes that occur during resistance exercise in fasted, untrained young men (Deldicque *et al*, 2008). It is well accepted that during hypertrophy following resistance exercise, PKB phosphorylation at Thr308 is seen to increase and is in fact required for an increase in muscle mass and size (Schiaffino and Mammucari, 2011; Luciano *et al*, 2017). This occurs as part of the traditional "Akt-mTOR" axis of signalling, though as noted by Bodine (2006) endurance exercise has been known to phosphorylate PKB in the absence of any change in mTOR1 signalling. In the fed state, using resistance trained, male participants it was shown that no significant change to PKB phosphorylation in response to resistance exercise alone, resistance exercise followed by endurance exercise or endurance exercise followed by resistance exercise occurred, when measured 1hr post-exercise (Jones *et al*, 2016).

The next most internally consistent set of changes were seen with cells stimulated with the 1EX protocol. In this case the significant reduction in P70S6K phosphorylation was accompanied by the expected decrease- though not significant- in mTOR phosphorylation. If it is accepted,

based on the matching depression in mTOR and P70, that myotubes had entered a period of reduced protein synthesis then it would be expected that 4ebp1 phosphorylation would be low, as this is the state in which 4eBP can bind and sequester EiF4F (Dreyer *et al*, 2006). EiF4F, also referred to as the cap binding protein, facilitates the alignment of incoming mRNA with the ribosome, which occurs in many cell types (Batool *et al*, 2019). In muscle specifically, transgenic mice with constitutively active 4ebp1 (always bound to EiF4F) showed unimpeded response to insulin when on a high fat diet. Additionally, they showed significantly lower blood glucose despite a lower lean mass than control mice, without a reduction in total muscle protein synthesis (Tsai *et al*, 2015). The removal of the initiation factor by binding is associated with autophagy and a number of beneficial adaptations including increased mitochondrial function and improved cardiac remodelling in mice with cardiomyopathy (Jin *et al*, 2020). Increased autophagy as a response to exercise is a known occurrence and could explain some of the changes seen here in a manner that is consistent with the known functions of 4ebp1 and its binding target in humans (Brandt *et al*, 2018). For the most part 4EBP phosphorylation remained unchanged in the 1EX EPS protocols, though the 1E6 frequency did produce a significant decrease. It is important to note however that measurements from the 1E6 frequency effectively represent 5 hours post exercise. This is important as Nikolic *et al* (2018) mention, the effects of EPS are dependent on the duration of stimulation, the pulse frequency as well as the time since the last "bout". It should be remembered that the depression of P70 phosphorylation cannot easily be explained as exercise induced suppression of protein synthesis for the conservation of energy, as EPS had long since ceased by the time of harvesting, unlike the previous chapter's experiments.

The 2EX EPS protocol produced results that were less consistent. The only significant change in P70 phosphorylation took place in response to 2E6, but this did not come with a reduction in

mTOR activity. Despite this, 4EBP1 was phosphorylated to a high degree. In this configuration, P70 and mTOR would not be increasing anabolic signalling, whilst 4EBP1 would not be inhibiting initiation of translation. This could be due to mTOR-independent regulation of 4EBP1 as previously proposed, which is a factor that is receiving more interest in recent years (Hodson *et* al, 2019). Another possibility is that the difference between 1 hour of activity every 6 hours and 2 hours of activity every 6 hours crosses a threshold for recovery, and that by reapplying EPS during the recovery period, the response to the stressor is not beneficial and hypertrophic, but deleterious (Kramer and Goodyear, 2007). However, this explanation seems unlikely given that the EPS settings used have been applied chronically for 24 hours and that EPS of the same setting was used as a "non-damaging" control for a study inducing damage via contraction (Orfanos *et al*, 2016). In that study, the damage that was identified was speculated to be a compensatory adaptation to muscle stress, which would both lessen future stress within the same bout and form part of the normal response to exercise. In other words, it should not induce abnormal effects in the signalling profile.

The only way in which 3EX matched 1EX and 2EX protocols was the lack of significant change in mTOR activity. However, this did not prevent a decrease in P70 phosphorylation by over half for 2 of the EPS frequencies- 3E12 and 3E8. This only partially overlapped with the changes seen in 4ebp1 phosphorylation, in which 3E24 and 3E12 seemingly induced a ~1.7-fold increase in phosphorylation. Half of this change (P70 decrease) suggests reduced anabolism, whilst the other half suggests increased anabolism. Given that increased 4ebp1 phosphorylation is indicative of translation initiation, additional blots of EEF2 may be useful to determine if that initiation is proceeding to elongation. The lack of p-P70 would suggest low activity of ribosomal S6- a protein whose activation is associated with increased cell size (Ruvinsky *et al*, 2005; Gombos *et al*, 2021).

Though a number of explanations have been offered by this point, when considering all of the results here, particularly glucose uptake and signalling changes it seems most likely that after the initial bout of EPS, sarcomeres rapidly disassembled. This conclusion seems more likely than depletion of nutrients, since we know that the media provided enough amino acids and glucose to sustain 24-hours of continuous contraction. Though the same paper that demonstrated their rapid disassembly also showed they reform under EPS, it could be the case that the downstream signalling did not recover in the same manner, possibly being susceptible to diminishing returns from the following bouts as seen in Kotani *et al (2021)*, though that experiment used EPS of live animals designed to elicit maximal tetanic contraction.

3.3.3 Limitations and Improvements

A key difficulty for interpreting the data generated by this experiment was the variable time between the end of contraction and harvesting. This was necessary to provide the context of a 24-hour cycle to the resulting uptake of glucose generated by contraction. The finding that none of the frequencies tested changed lactate, glycogen (except 1E6), or glucose uptake significantly from baseline could be attributed to several factors. The rationale for choosing frequencies shown in **Figure 3.1** is that they could be compared on the basis of equal contraction time but different frequencies- such as 12E24 and 3E6, or on the basis of increasing EPS duration such as 1E24, 2E24 and 3E24. This was done in order to prevent confounding by the possibility that a potential difference could be rooted in the amount of work performed as measured in joules rather than due to some effect of the rest period between bouts (Petridou *et al*, 2019). Similarly, cells were not harvested immediately after the end of EPS because of the intention to draw a comparison between the intermittent EPS and 24-hour continuous stimulation. If cells had been harvested immediately after cessation of EPS then we would have

been unable to measure any lingering benefit to glucose uptake provided by EPS (Atherton and Rennie, 2006). However, for metrics other than protein phosphorylation, comparison to the unstimulated state became more appropriate, as there was not sufficient difference between the outcomes of the various frequencies to discern a real difference between the intermittent EPS and continuous EPS. The lack of significant difference between 3E6 and 12E24 protocols led us to conclude that the sarcomere disassembly mechanism was the most likely explanation for lack of glucose uptake, rather than missing the increase in uptake due to measuring hours after the end of contraction. We arrived at this conclusion based on the fact that whilst there was a 12-hour gap between cessation of EPS and harvesting for 12E24, the same gap for 3E6 was only 3 hours and yet the difference in glucose uptake was negligible. Combined with the inability of 2DG6P to escape from the cell or be reduced by metabolism, we suggest that uptake was not elevated at any point. Given the linear uptake of glucose demonstrated in the previous chapter and the fact that glucose uptake shown is normalized to total protein we would have expected 12 hours of stimulation to produce an increase in glucose uptake half that of 24 hours at 2.5µM, but the actual value was lower at only 1.6µM.

A suggested refinement of this experiment would reduce the number of contraction patterns tested and introduce harvesting points at 30 minutes, 2 hours and 3-5 hours post EPS. This would require adjustment of the protocols selected (and therefore also the premise of the 24-hour window) in order to collect the 5-hour post EPS timepoint, which could land 11-15 hours after the start of the experiment, which would be difficult for a single researcher to manage. This would reduce the unknown factor of changes to signalling that occur after the end of EPS. Additionally, a 0.1Hz maintenance pulse would be used in between “experimental” EPS bouts to minimize or prevent disassembly of sarcomeres.

3.4 Conclusion

Using the methods outlined above, we were unable to identify a point of diminishing returns in either glucose uptake or anabolic signalling in response to intermittent EPS. Flaws in the assumptions made during the conception of the experiment resulted in difficulty in decisively ascribing the lack of change in glucose uptake, lactate output and glycogen that we reported to a specific cause. We did, however, record changes in anabolic signalling, however, we again had difficulty in establishing a rationale for the changes or lack thereof in phosphorylation of P70, 4EBP1 and mTOR. PKB phosphorylation behaved in a manner consistent with literature description, showing a reduction in response to all contraction patterns, however a reliable pattern in response to changes in frequency or duration could not be established within this observed reduction. We highlighted two primary areas for improvement in the design of this experiment for future researchers, namely the introduction of a maintenance EPS pulse to prevent sarcomere disassembly and the suggestion that multiple harvesting points after the end of EPS be introduced.

Chapter 4: Establishing a disuse phenotype *in vitro* using CPA and blebbistatin.

4.0 Introduction

4.0.1 Impact of disuse on muscle

Skeletal muscle undergoes several changes when faced with extended periods of disuse. This can be as a result of an immobilizing injury, sedentary lifestyle or exposure to microgravity. These are a reduction in Strength and CSA (Cross sectional area) (Brian, 2009), which are underpinned by a reduction in the rate of protein synthesis. Part of this change includes a reduction in the sensitivity of muscle to the hormone insulin, which is critical for normal glucose uptake as well as anabolic / anti-catabolic signalling (Fujita *et al*, 2006). As result of insulin resistance, glucose uptake into the muscle slows, resulting in increased glucose in other compartments, most notably the blood. Prolonged elevated blood glucose results in the formation of AGEs (Advanced Glycation End products), which result in abnormal functioning of the effected proteins, which is known to be involved in the commonly observed vascular damage associated with diabetes (Vlassara and Uribarri, 2014).

One of the major changes that underpin the macro scale reduction in CSA and strength are dysregulation of calcium signalling, specifically a sustained elevation in cytoplasmic calcium ion concentration at a sub contractile level (Shur *et al,* 2021). This is known to alter the regulation of many proteolytically active enzymes, which could have important implications in the muscle synthesis/ degradation balance as well as in the mechanisms responsible for recruitment of GLUT4 to the plasma membrane.

The second major change is the lack of mechanically induced signalling brought about by physical activity. Removal of this stimuli removes much of the impetus for activation of pro-synthetic pathways and muscle hypertrophy. It also has the effect of reducing ATP

requirements, which is suspected to have effects on maintenance of mitochondrial quality and glucose uptake (Sorriento *et al*, 2021).

4.0.2 Limitations of *in vivo* study of muscle disuse

Two models of microgravity- dry immersion and head tilted bedrest- have shown that a high degree of loss of strength occurs over28 - 55 days of muscle disuse- 32.9% (Blottner *et al*, 2006) and 46% (Duchateau, 1995)- which is similar to that seen in intensive care unit patients (Parry and Puthucheary, 2015). However, the underlying changes that occur within each myotube to produce these changes happen much more quickly and can be detected in as little as 72 hours. The problem with detecting and assigning meaning to these changes *in vivo* is that they occur concurrently with many other changes in signalling, making it unclear what is an adaptation to changing environment and what is a compensatory response to adverse conditions. Additionally, signalling at the tissue and whole-body level can have a significant effect on the changes that occur within muscle, further complicating interpretation of cause and effect. For example, abnormally functioning neuronal tissue can result in muscle which is severely weakened, due to a lack of trophic factors that are received from nerve tissue (Saini *et al*, 2021). Adipose tissue can secrete adipokines which are pro inflammatory, leading to insulin resistance in muscle (Stanford and Goodyear, 2018). Whilst it has never been decisively proven to occur in humans, “training” or browning of fat in response to exercise, resulting in release of adipokines which improve the function of muscle has been observed to occur in rodents as a result of exercise. This complex loop of inter-tissue signalling makes determining muscle exclusive changes

more difficult and is therefore an argument for the development of muscle specific models.

4.0.3 Cell culture study of muscle disuse (include EPS)

There are a number of ways in which muscle pathologies can be simulated in a cell culture environment, ranging from cell lines derived from individual donors who suffer from a specific condition, such as muscular dystrophy (Caviedes *et al*, 1994), to genetic knockout or knockdown of target genes (Soblechero-Martín *et al*, 2021), as well as drug induced recreation of pathological events.

Drug induced simulation of diabetes and muscle wasting have previously been used in whole animals, such as by injection with streptozotocin (STZ), which causes failure of the pancreatic islets, which then initiated downstream effects that lead to type 1 diabetes (Furman, 2021). This relies on the relatively specific toxic action of STZ rather than acting through signalling pathways, so is not suitable for single cell models. Similarly, glucocorticoid drugs have been used to create a diabetes like state in rats (Severino *et al*, 2002) and that in L6 cells includes muscle protein degradation, however, the action of dexamethasone in particular is known to be different from that of endogenous glucocorticoids, with models of sarcopenia induced by dexamethasone lacking upregulation of P70 and 4ebp1 seen in aged mice (Wang *et al*, 2023) An alternative model would be useful (Itagaki *et al*, 2010). Other methods of inducing diabetes-like metabolism in cell line based models include providing excessive lipid to cells, which has a similar effect that a high fat diet might have in animal studies. One reasons for discounting the use of lipids were that the effects would largely be localised

to changes to mitochondrial function, which whilst relevant, only make up part of the desired phenotype (Bakar and Tan, 2017). Additionally, growth in high glucose media, which is standard for C2C12 cells already induces a diabetogenic effect when compared to low glucose media (Luo *et al*, 2019).

One of the key benefits of a cell culture system for studying skeletal muscle is the ability to apply a controlled bout of activity by means of electrical pulse stimulation (EPS). Unlike rodents allowed to run at will, which can be confounded by stressors like housing conditions and handling stress, cells undergoing EPS should have a consistent response between wells as conditions are identical. The benefit of using C2C12 cell specifically for this purpose is their high contractile ability compared to human and L6 cells as well as their relatively high stability when passaging, which is not always the case for human donor derived cells, which may or may not retain the phenotype (in this case exercise response) of the donor (Chang *et al*, 2014; Balci-Hayta *et al*, 2018). Of course, the advantage of simplified determination of cause and effect in a single cell model comes at the price of reduced physiological representativeness, given that muscle does not exist in isolation *in vivo* and that monocultured myotubes are typically not as mature as myotubes found in living tissue.

4.0.4 The use of Cyclopiazonic acid and Blebbistatin in studying muscle

In order to recreate the desired phenotype, we decided that manipulating physiological conditions to recreate two primary conditions, which are thought to be critical for the initiation of the typical changes associated with muscle disuse. The first condition is that

contraction should be inhibited. Contraction or -*in vivo*- exercise is a powerful stimulant for increasing glucose uptake (Flores-Opazo *et al*, 2020), pro-anabolic signalling (Cuthbertson *et al*, 2006) and is seemingly responsible for the maintenance of normal function in skeletal muscle, particularly mitochondrial function and recycling (Vainshtein *et al*, 2015). The second condition is the elevation of cytoplasmic Ca^{2+} which is known to occur as a result of long-term muscle disuse/ immobilisation (Sharlo *et al*, 2021; Hyatt and Powers, 2020). The importance of elevated cytoplasmic calcium in shaping the disuse phenotype is suggested to be activation of proteases that disassemble contractile elements within muscle. It has also been postulated that these proteases may exert influence on glucose uptake, given the importance of actin and myosin-based structures for trafficking GLUT4 bearing vesicles to the plasma membrane (Tammineni *et al*, 2020).

In order to prevent contraction, we have elected to use blebbistatin, a muscle specific inhibitor of myosin II ATPase. Blebbistatin inhibits contraction by preventing myosin-actin bridge crawling, which under ordinary conditions would allow sarcomeres to shorten. It does this by non-competitive inhibition of ATPase. Without this, the sarcomeres remain in a position in which myosin and actin are not connected via cross linking (Kovács *et al*, 2004; Rahman *et al*, 2018). By applying blebbistatin before CPA it is hoped that we can achieve a rise in the cytoplasmic calcium ion concentration without the accompanying contraction. Blebbistatin has been used previously in the study of muscle to demonstrate the necessity of cortical actin for differentiation of myoblasts to myotubes (Duan and Gallagher, 2009) and to prevent contraction induced glucose uptake in a study of the effect of calcium on glucose uptake (Jensen *et al*, 2014).

Importantly, blebbistatin alone is not thought to have an effect on calcium handling, making it suitable for decoupling contraction from calcium in these studies.

Cyclopiazonic acid (CPA) is a SERCA 2 inhibitor first isolated from *Aspergillus.* Its specific inhibition of SERCA is the property that makes consumption of food contaminated with CPA producing microbes dangerous as well as a useful tool for manipulation of calcium transients *in vitro* (Hutchet and Léoty, 1993; Ostrey *et al*, 2017). Crystallography and computer modelling of the binding site suggests that CPA inhibits the re-uptake of calcium ions from the cytoplasm by blocking the channel (RyR) linking the sarcoplasmic reticulum (SR) to the cytoplasm, which is achieved by binding at or near Phe^{256} (Moncoq *et al*, 2007). Without calcium re-uptake, over time the concentration of Ca^{2+} in the cytoplasm increases, eventually leading to sustained contraction. This timescale is relatively short, in the order of seconds to minutes, as CPA has been used to force contractions to occur in muscle fibres (Rose *et al*, 2009; Même *et al*, 1998). Additionally, cytoplasmic calcium ion concentration can be lowered by diffusion to the extracellular surface of the plasma membrane, if the extracellular concentration causes a gradient to form. CPA has previously been used to prevent the reuptake of Ca^{+} into C2C12 cells in order to study caffeine and nicotine induced calcium transients, with the finding that there were differential transients in mitochondrial calcium between the two (Challet *et al*, 2001). It has also been successfully used to elicit an increase in cytoplasmic calcium when testing the properties of the RyR in a calcium free media, in which it was found that there was very little loss of calcium to the extracellular media from the cytoplasm (Filip *et al*, 2019).

Both CPA and blebbistatin are poorly soluble in water, necessitating solutions of DMSO. Not only do both of these solutions have short shelf lives, but at sufficiently high doses and lengths of exposure, will exhibit toxic effects on cells (Rauscher *et al*, 2018; Norred *et al*, 1985). Additionally, blebbistatin can exhibit phototoxic effects in response to visible light, particularly blue light wavelengths, which are not proportional to the length of the light exposure and rapidly induce cell death even at low doses (Kolega, 2004). For this reason, it is important that before and after administration of the drugs, vials and plates should be shielded from natural light.

4.0.5 Aims and hypothesis

Two of the major pathological hallmarks of disuse atrophy at the cellular level are elevated cytoplasmic calcium and reduced physical activity. In order to recreate the downstream phenotype generated by chronic exposure to these conditions we aimed to impose them through the addition of CPA and blebbistatin. CPA is a SERCA pump inhibitor. By inhibiting the re-uptake of calcium to the sarcoplasmic reticulum we intended to raise the cytoplasmic concentration of calcium. Blebbistatin is an inhibitor of myosin II ATPase, which binds to the ATPase and locks it in the tension free state, preventing actin-myosin bridge crawling. We hypothesize that with the application of both of these drugs, conditions would be created that would lead to reduction of glucose uptake and changes in signalling that favoured breakdown of contractile elements rather than synthesis.

4.1 Methods

4.1.1 Cell culture

Cell culture of C2C12 cells was performed as described in chapter 2. Cells used for confirming the effect of blebbistatin, CPA and DMSO on viability were of passages 8 to 10. Seeding vials originated from the same stock. For the purposes of this chapter, n=6 refers to 6 wells of a plate.

4.1.2 EPS protocol

EPS was performed on fully differentiated myotubes using an Ionoptix C-Pace EP (IonOptix, Milton, MA, USA) connected to two 6-well compatible C-dishes, also from Ionoptix. Before each use, the electrodes were drained of waste build up and sterilized with ethanol before rinsing with dH_2O. The pulse train settings were as follows: 11.5V, at a frequency of 1Hz with each pulse lasting 2ms, informed by previous studies which used the same settings and saw release of exercise associated cytokines (Evers-van Gogh *et al*, 2015). Similar pulse train settings also caused uptake of glucose (Nikolic *et al*, 2917). A volume of 2ml media per well was sufficient to ensure electrodes were submerged. Cells were subjected to 1 hour of pre- stimulation to allow time for functional sarcomeres to form. Pre-stimulation used the same pulse train settings as the experimental EPS, 11.5V, 1Hz, 2ms. After pre-stimulation, any treatments were added, and cells then received 24 hours of EPS.

4.1.3 Drug treatment protocol for the establishment of a disuse phenotype

C2C12 cells were cultured until mature myotubes were formed at day 5, at which point they received a final media change (from differentiation media into fresh differentiation media). Twenty-four hours after this media change, either blebbistatin alone, CPA alone, 100% DMSO, C+B in 100 %DMSO or C+B 27% DMSO were added to cells, which were then returned to the incubator, without electrical stimulation for 24 hours. After this period, the cells were harvested as described in previous chapters. The final concentration of blebbistatin used was 10µM, whilst the final concentration of CPA used was 100µM, both of which were chosen on the basis of the efficacious dose and concerns over toxicity due to the volume of DMSO required to solubilize the concentration of blebbistatin required. For this reason, initial experiments ("blebbistatin only") with blebbistatin use 30mM blebbistatin in 100% DMSO whilst later experiments using both blebbistatin and CPA use only 10uM blebbistatin in a 27% DMSO solution. Literature suggests (Rauscher *et al*, 2018) that muscle sensitivity to blebbistatin is sufficiently high that a response would be seen at doses as low as 0.1-1uM.

4.1.4 Viability assay

Cell viability was assayed using resazurin. This was necessary thanks to the use of DMSO as a vehicle, but also because both CPA and blebbistatin are known to be toxic at high does and in the case of blebbistatin to produce toxic metabolites during photo-degradation. After treatment with drugs for 24 hours, cell culture media was removed and replaced with a sterile filtered 10µg/ml solution of resazurin in HBSS, made from a stock solution of 100µg/ml. Cells were returned to the incubator for one hour to allow the dye to enter the cells and be reduced from resazurin to resorufin. The detection of the reduction product was achieved by measuring

fluorescence with an excitation of 530nm and an emission filter of 590nm. For measurement, the resazurin/resorufin mixture was gently mixed to ensure uniformity and 100µl taken off in triplicate from each well of cells and added to a 96 well plate with black walls. The plate was then measured without the lid attached.

4.1.5 Lactate assay

Measurement of media lactate concentration was performed as described in chapter 2

4.1.6 Glycogen assay

Measurement of cell glycogen was performed as described in chapter 2

4.1.7 Glucose uptake

Measurement of glucose uptake as internal 2DG6P was performed as described in chapter 2, with slight modification. Rather than performing 1 injection of 6 separate samples, 3 samples were used for each timepoint, injected twice. This modification was made to reduce mass spectrometer running time and cell culture requirements.

4.1.8 Measurement of signalling

Anabolic signalling was measured by western blotting as described in chapter 2. Additionally, targets relating to glucose uptake and metabolism were blotted- pERK1/2 (S217/221)), and pEEF2 (T56) pPKB (T308).

4.1.9 qPCR

Messenger RNA was recovered by extraction using Trizol reagent (Invitrogen, Carlsbad, California, US) and chloroform, following the manufacturer instructions, without the use of

Target-Direction	Sequence (5'-3')
GAPDH-F	ACTCCACTCACGGCAAATTC
GAPDH-R	TCTCCATGGTGGTGAAGACA
CALM1-F	GTGGTGCCGTTACTCGAAGTC
CALM1-R	GACTTGTCCGTCGCCATCAA
ATP2A1-F	GACGAGTTTGGGGAGCAGCT
ATP2A1-R	AGGTGGTGATGACAGCAGG

Table 4.1: Primer sequences used for qPCR. Targets are biomarkers known to become upregulated in response to elevated calcium.

glycogen. cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, 4368814). qPCR was carried out using a 7900 HT fast real-time PCR system (Applied biosystems, Foster City, California, US) in order to determine the change in expression of CALM1 and ATP2A1. These genes are known to show upregulation of mRNA transcripts even during relatively short human bedrest protocols. Melt curves were run to confirm that only a single product was present. In the event of unclear results from the melt curve a gel was run using amplification products from the PCR plates.

4.1.10 Data analysis

Data analysis was performed as described in chapter 2.

4.2 Results

4.2.1 Viability assay

A dose response curve of DMSO against viability as a percentage of an untreated control was established to determine the maximum permissible DMSO volume that could be used to suspend blebbistatin and CPA. Four volumes of DMSO corresponding to 0.14%, 0.28%, 0.57% and 1.15% v/v were added to cells. No dosages resulted in a significant change from the untreated control, however, the highest dose- 1.15%- produced a slight decline in viability to 96.6%, which caused it to differ from all of the lower doses. The lower doses induced a slight but non -significant increase in viability as measured by oxidation which is depicted in **Figure 4.1** (1.15% vs 0.57% p= 0.0006, 1.15 vs 0.28 p= 0.0003, 1.115% vs 0.14% p= 0.0305).

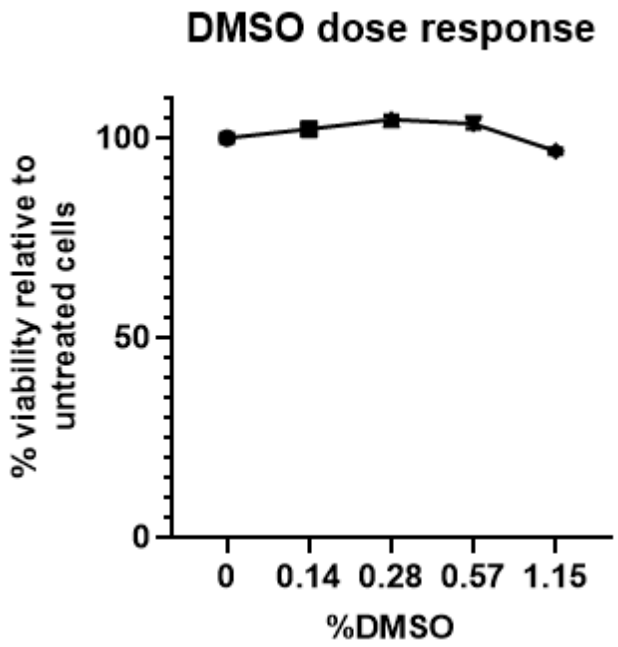


Figure 4.1: A dose-response curve showing the effect of increasing DMSO concentration on cell viability as determined by oxidation of resazurin to resorufin. N= 3. Error bars indicate SEM.

4.2.2 lactate assay

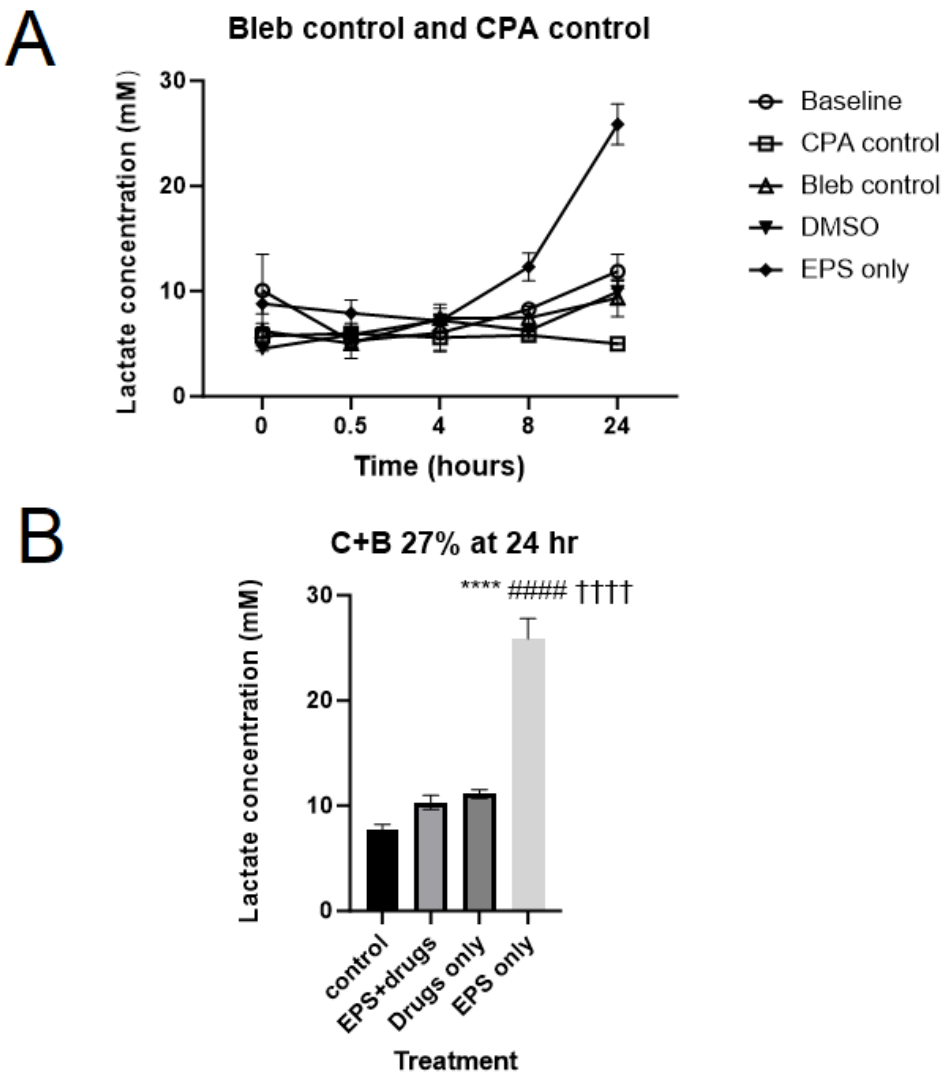


Figure 4.2: A) shows the effect of various single drug treatments as well as a vehicle control on media lactate over time. n= 3. Bars indicate SEM. B) Graph of media lactate after 24 hours of treatment with both Blebbistatin and CPA or blebbistatin, CPA and EPS. n= 6.

Measurement of media lactate from cells that received either no treatment, treatment with CPA alone, Blebbistatin alone or DMSO alone showed that over the 24-hour period of exposure there was no significant difference between treatments. EPS alone significantly increased media lactate from baseline values at 24 hours (p= 0.0091). For cells treated with either CPA + Blebbistatin or CPA + blebbistatin and EPS, after 24 hours, there was a slight increase in media lactate, not reaching statistical significance. EPS alone saw an increase in media lactate up to 26.9mM, which was significantly higher than any of the other treatments (p <0.0001 in all cases)(**Figure 4.2**).

4.2.3 Glycogen assay

Comparison of cellular glycogen concentrations shows that there was no difference between cells treated with CPA alone or DMSO alone (**Figure 4.3**). Cells treated with blebbistatin did not differ from controls cells except at 24 hours (p=0.0072). At all timepoints EPS treated cells had significantly higher glycogen content than baseline cells (p= 0.0011, 0.0014, 0.0048, 0.0054, 0.0037 at 0, 0.5, 4, 8, 24 hours respectively). Though there were differences between the individual treatments over time, within each treatment there was no effect over time. For cells treated with either CPA+ blebbistatin or CPA +blebbistatin+ EPS there was no significant difference from control cells after 24 hours. Cells treated with EPS alone had significantly higher glycogen content, representing approximately a 4-fold change from control and a 2-fold change from

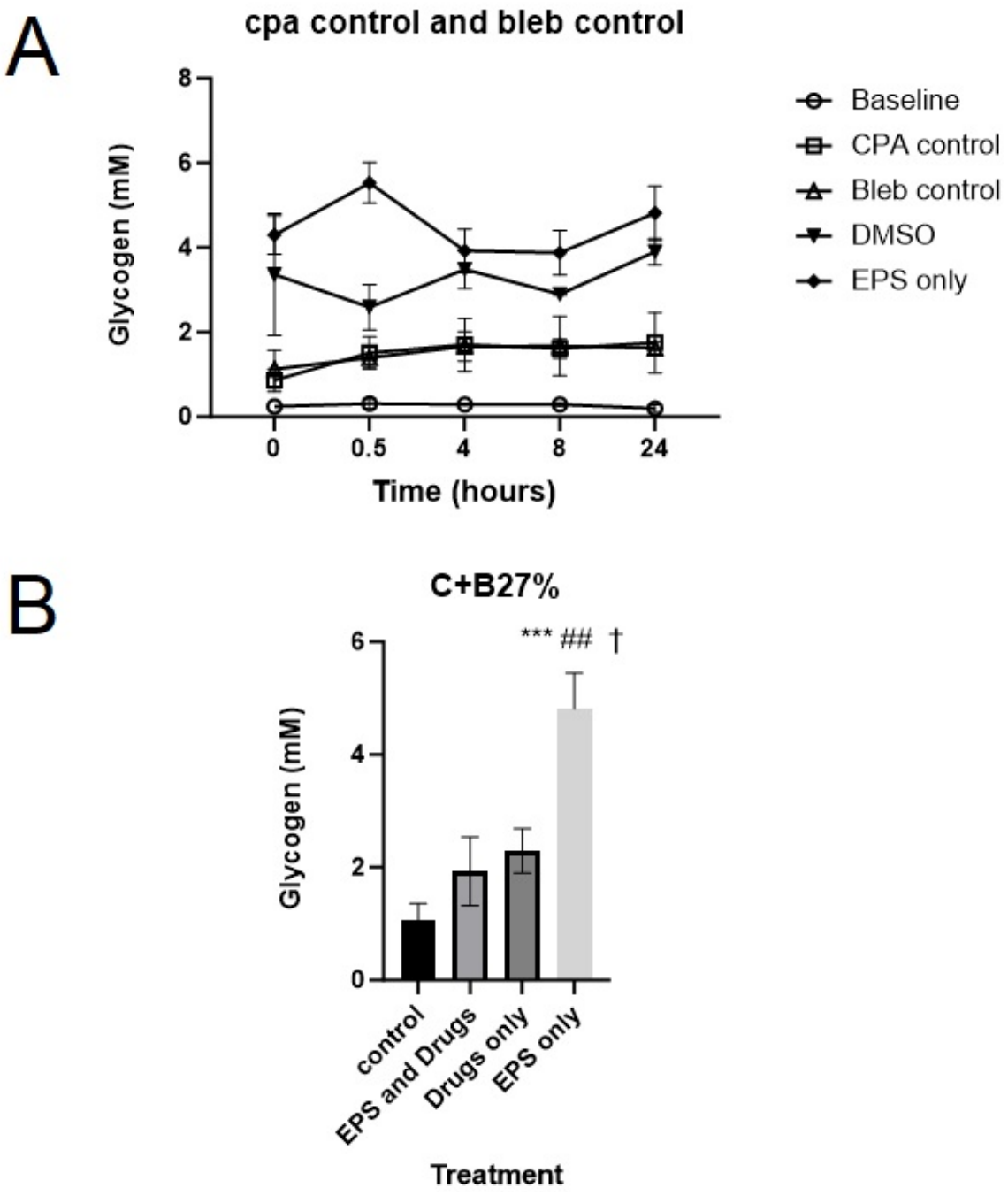


Figure 4.3: A) Showing changes in cellular glycogen concentration over time in response to individual drug treatments. B) showing an increase in glycogen with treatment with drugs alone and with a combination of drugs and EPS. n=3. Error bars indicate SEM.

either treatment with CPA and blebbistatin (p= 0.0005 control vs EPS, p= 0.0053 EPS vs Drugs+ EPS, p= 0.0225 EPS vs Drugs only).

4.2.4 Signalling changes

A vehicle control of DMSO at a concentration of 1.15% v/v was run in order to differentiate the effect of the drugs CPA and blebbistatin from the solvent. mTOR phosphorylation showed a steady increase over 24 hours, reaching a 2.9-fold increase above baseline by 24 hours (p=0.0302). This pattern was reflected in the phosphorylation of P70, however, the only significant change was an initial decrease in phosphorylation at 30 minutes post- treatment (p= 0.0336), after which phosphorylation increased steadily until reaching 1.7-fold greater than baseline, which was not statistically significant. EEF phosphorylation fluctuated over the course of 24 hours but were not significant owing to a high degree of variance. Significant increases in PKB phosphorylation were seen at 30 minutes and 4 hours (p=0.0135 and 0.0076), reaching a 2.8-fold increase, which returned to baseline by 24 hours (**Figure 4.4**).

Cells treated with blebbistatin (**Figure 4.5)** showed no significant changes in signalling related to muscle anabolism. Similarly, EEF2 phosphorylation did not change significantly, spiking at 4 hours at 1.8fold greater than baseline. PKB phosphorylation also did not change significantly, though it did appear to increase at 8 and 24 hours post treatment. The only significant change recorded due to application of blebbistatin was ERK1/2 phosphorylation, which dropped sharply at 8 and 24 hours to a half and a quarter of the baseline value respectively. The change at 8 hours was only significantly different from the 4hr value (p=0.0430), whilst the change at 24 hours was significantly different from both baseline and 4hrs (P= 0.0244 and 0.0077 respectively).

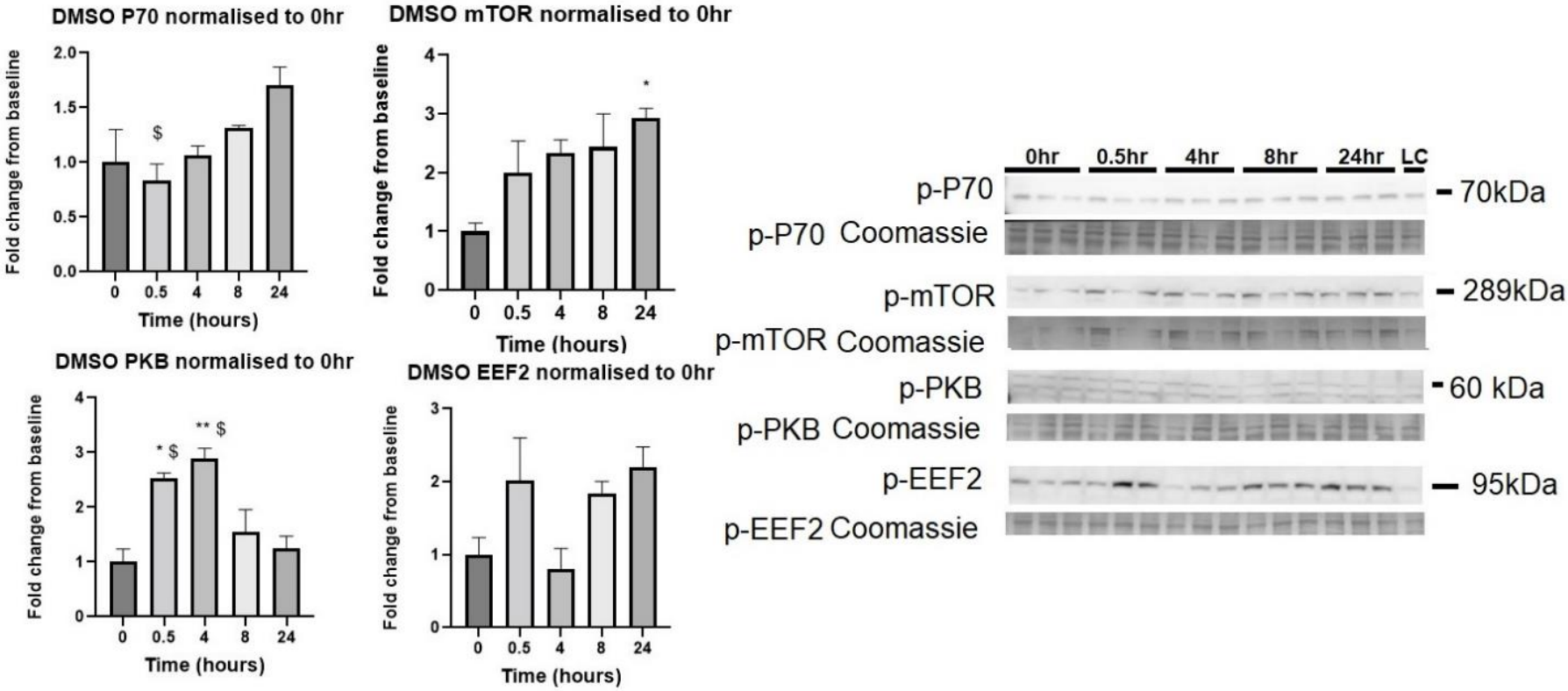


Figure 4.4: Data with representative blots and Coomassie stain. C2C12 myotubes were treated with 1.15%v/v DMSO for 24 hours and then harvested for western blot preparation. * indicates a significant difference from baseline (0hr). $ indicates a significant difference from the 24hr timepoint's value. N= 3. Error bars indicate SEM.

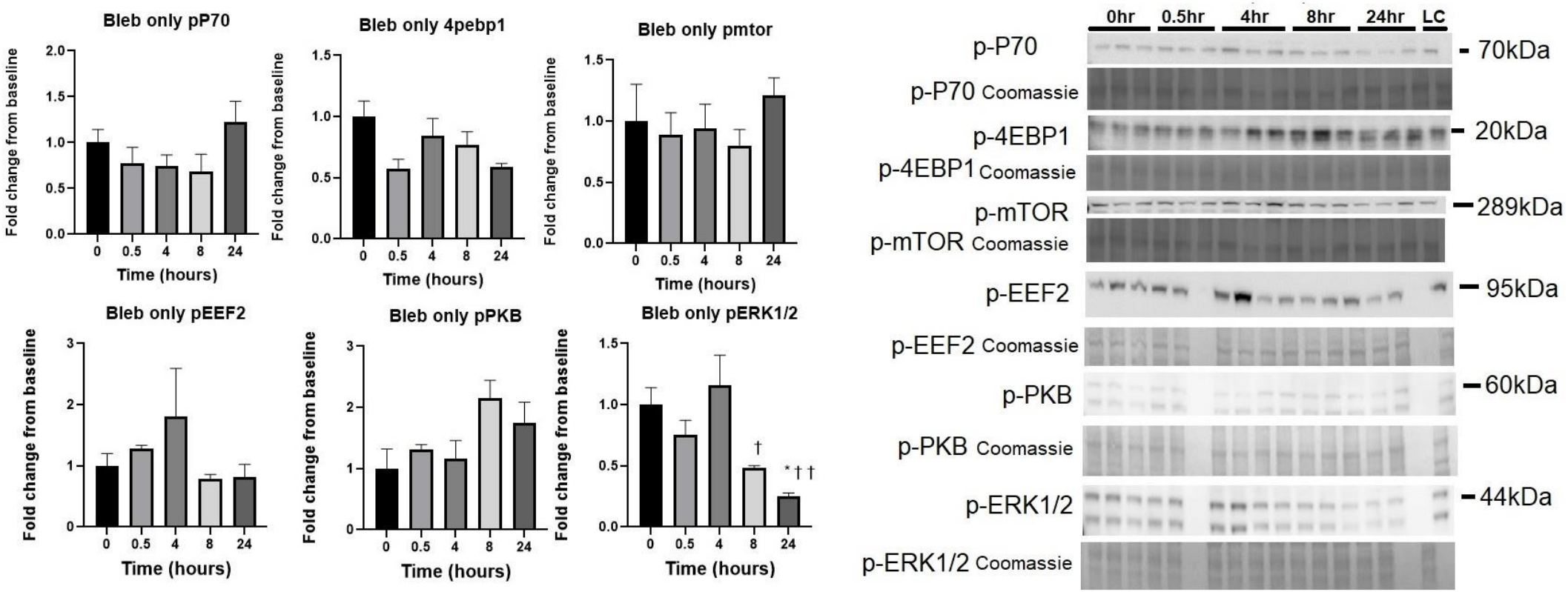


Figure 4.5: Western blot data (See appendix A for representative blots and Coomassie stain). C2C12 myotubes were treated with 30uM Blebbistatin in DMSO (final conc 1% v/v) for 24 hours and then harvested for western blot preparation. * Indicates a significant difference from baseline (0hr). $ indicates a significant difference from the 24hr timepoint's value. ǂ indicates significant difference from 4hr value. N= 3. Error bars indicate SEM.

Cells treated with cyclopiazonic acid alone showed a steady increase in mTOR phosphorylation over 24 hours, which peaked at a 1.8-fold increase. Though this was not statistically significant, it was associated with changes to both 4EBP1 and p70 signalling. P70 signalling peaked at a 1.5-fold increase, which was not significantly more than baseline, but was significantly greater than the value recorded at 24 hours, which was only 34% of baseline (p=0.0024). There was no difference from baseline in 4ebp1 signalling, but there was a difference between values at 4 and 24 hours, which were 1.4-fold and 0.8-fold of baseline respectively (p= 0.0216) (**Figure 4.6**). CPA treated cells also showed decreasing phosphorylation of EEF2 until the 8-hour point. At 24 hours phosphorylation had increased to 1.5-fold baseline. This was not significantly greater than baseline, but was significantly greater than 0.5hr, 4hr and 8hr (p= 0.0428, 0.0072 and 0.0028 respectively). Neither PKB or ERK1/2 phosphorylation changed significantly though ERK1/2 phosphorylation had fallen to 51% of the baseline value by 24 hours.

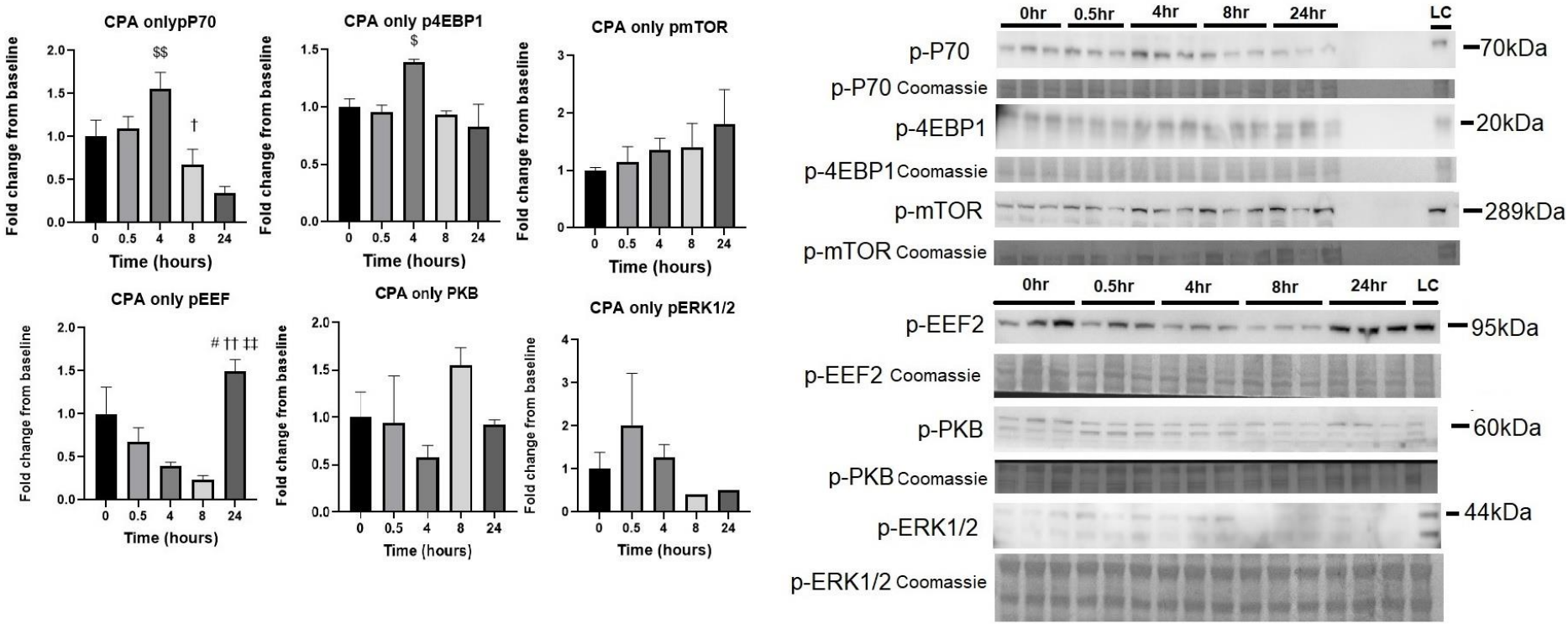


Figure 4.6: WB data. C2C12 myotubes were treated with 100uM Cyclopiazonic acid in DMSO (final conc 0.175% v/v) for 24 hours and then harvested for western blot preparation. * Indicates a significant difference from baseline (0hr). † indicates a difference from 4hrs. ‡ indicates a difference from 8hrs. $ indicates a significant difference from the 24hr timepoint's value. N= 3. Error bars indicate SEM.

Myotubes treated with both 30uM blebbistatin and 100uM CPA showed minor changes to anabolic signalling with no change in mTOR and 4EBP1 phosphorylation. P70 signalling increased at the 4-hour timepoint, showing a 1.5-fold increase over baseline (p= 0.0152). This change was not significantly greater than baseline, but it was significantly greater than the value recorded for 24 hours, which was 60% of the baseline value. EEF2 signalling was decreased between 0.5hours and 8 hours, but not significantly so. At 24 hours EEF2 phosphorylation increased 1.9-fold above baseline values, making it significantly different from all other timepoints (vs 0hr p= 0.0482, vs 0.5hr p= 0.0011, vs 4hr p= 0.0027, vs 8hr p= 0.0006). PKB phosphorylation showed an inverse trend, with signalling increasing between 0 and 4 hours (p=0.0054 and 0.0004), peaking at a 5.4-fold baseline increase before dropping sharply at 8hrs and 24hrs, reaching a final low of 25% baseline, which though not significantly less than baseline, was different from the values at 0.5hrs and 4hrs (p=0.0011 and 0.0001 respectively). ERK1/2 phosphorylation showed the same pattern as PKB, increasing until 4 hours, after which values decrease to levels below baseline. Only the 1.8-fold increase at 4 hours was significantly different from baseline (p=0.0385), whilst the decrease at 8hrs to 48% baseline was significantly different from 0.5hrs and 4hrs (p=0.0153 and 0.0019) but not baseline or 24 hours (**Figure 4.7)**

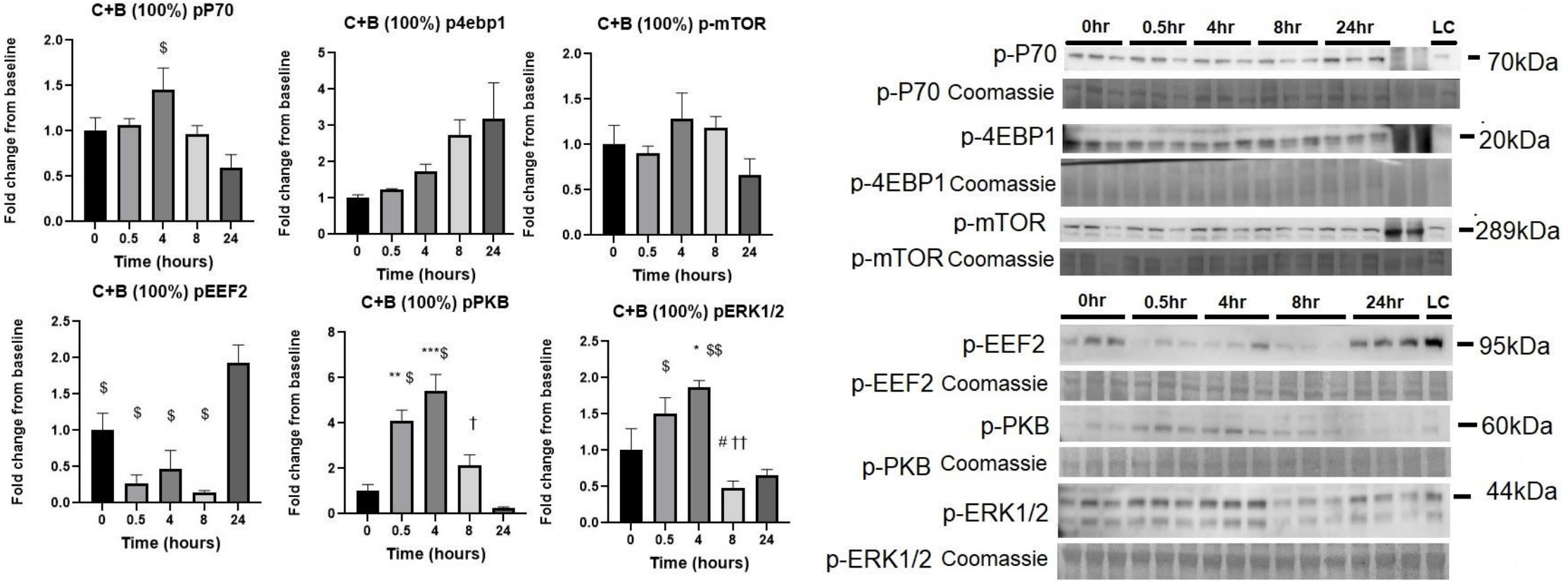


Figure 4.7: WB Data (See appendix A for representative blots and Coomassie stain). C2C12 myotubes were treated with both 30uM Blebbistatin and 100uM CPA DMSO (final conc 1.175% v/v) for 24 hours and then harvested for western blot preparation. * Indicates a significant difference from baseline (0hr). # Indicates a difference from 0.5hrs. † indicates a difference from 4hrs. ‡ indicates a difference from 8hrs. $ indicates a significant difference from the 24hr timepoint's value. N= 3. Error bars indicate SEM.

The final treatment group, cells treated with 100µM CPA and 10 µM blebbistatin in 27% DMSO (final DMSO concentration of 0.445%) was only measured at 24 hours post treatment and cells received either drugs alone or drugs in combination with continuous EPS (11.5V, 1Hz, 2ms). In this experiment, both cells treated with drugs alone and with drugs and EPS showed a significant ($p=0.0479$ and 0.0148 respectively) decrease in p70 (T389) phosphorylation when compared with unstimulated, untreated cells, but not when compared with cells that had received EPS alone. Phosphorylation of 4EBP1 increased markedly in response to drug treatment, with and without EPS. This was significantly ($p<0.0001$ for both) different from baseline and from EPS alone, which itself showed significantly less 4ebp1 phosphorylation when compared to control cells ($p=0.0070$).

As with previous experiments there was no significant change in mTOR phosphorylation, though EPS treated cells had much less phosphorylation than the other 3 treatments, often below the limit of detection (**Figure 4.8**). EEF2 phosphorylation increased in response to drug treatment alone ($p=0.0326$) and EPS alone (0.0016), but not drug treatment with EPS. EPS alone showed the greatest increase in pEEF2 signalling at 2.8-fold greater than control.

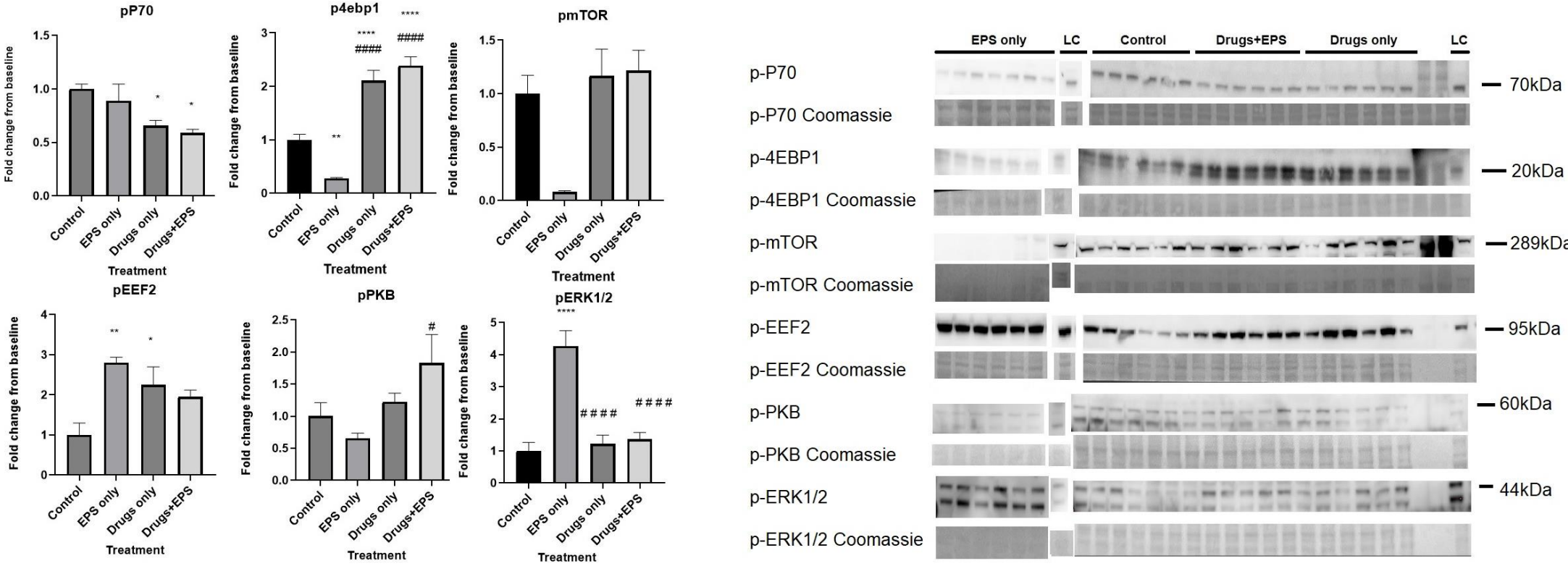


Figure 4.8: WB Data C2C12 myotubes were treated with both 10uM Blebbistatin and 100uM CPA DMSO (final conc 0.445% v/v) for 24 hours and then harvested for western blot preparation. * Indicates a significant difference from control. # Indicates a difference from EPS only. † indicates a difference from Drugs + EPS. N= 6. Error bars indicate SEM.

Changes in PKB phosphorylation compared to baseline were minimal, with only Drugs+EPS showing a significant change, increasing 1.8-fold ($p=0.0218$). Lastly, ERK1/2 phosphorylation did not change significantly from baseline in response to drug treatment, however, drug treatment prevented the 4.3-fold increase associated with EPS only. The p values for differences between EPS only and control, Drugs+EPS and Drugs alone were all <0.0001.

4.2.5 qPCR

Expression of biomarker genes ATP2A1 and CALM1 both increased in response to CPA and blebbistatin treatment for 24 hours, however, only the increase in CALM1 proved to be significant, with a p value of 0.0401 for control vs drugs and EPS, whilst for control vs drugs only $p= 0.0182$. The difference in value between the two treatments - with and without EPS were not significant for either marker.

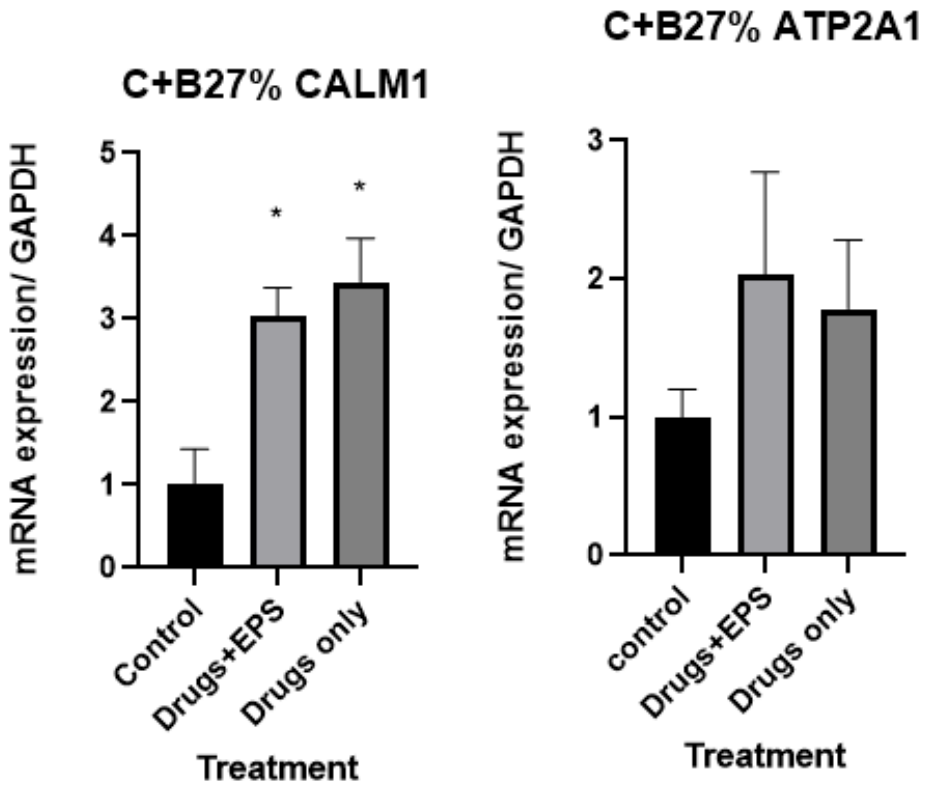


Figure 4.9: Changes in mRNA expression relative to untreated control for biomarkers known to increase in response to elevated cytoplasmic Ca^{2+}in skeletal muscle. N=3. Error bars indicate SEM.

3.5 Glucose uptake

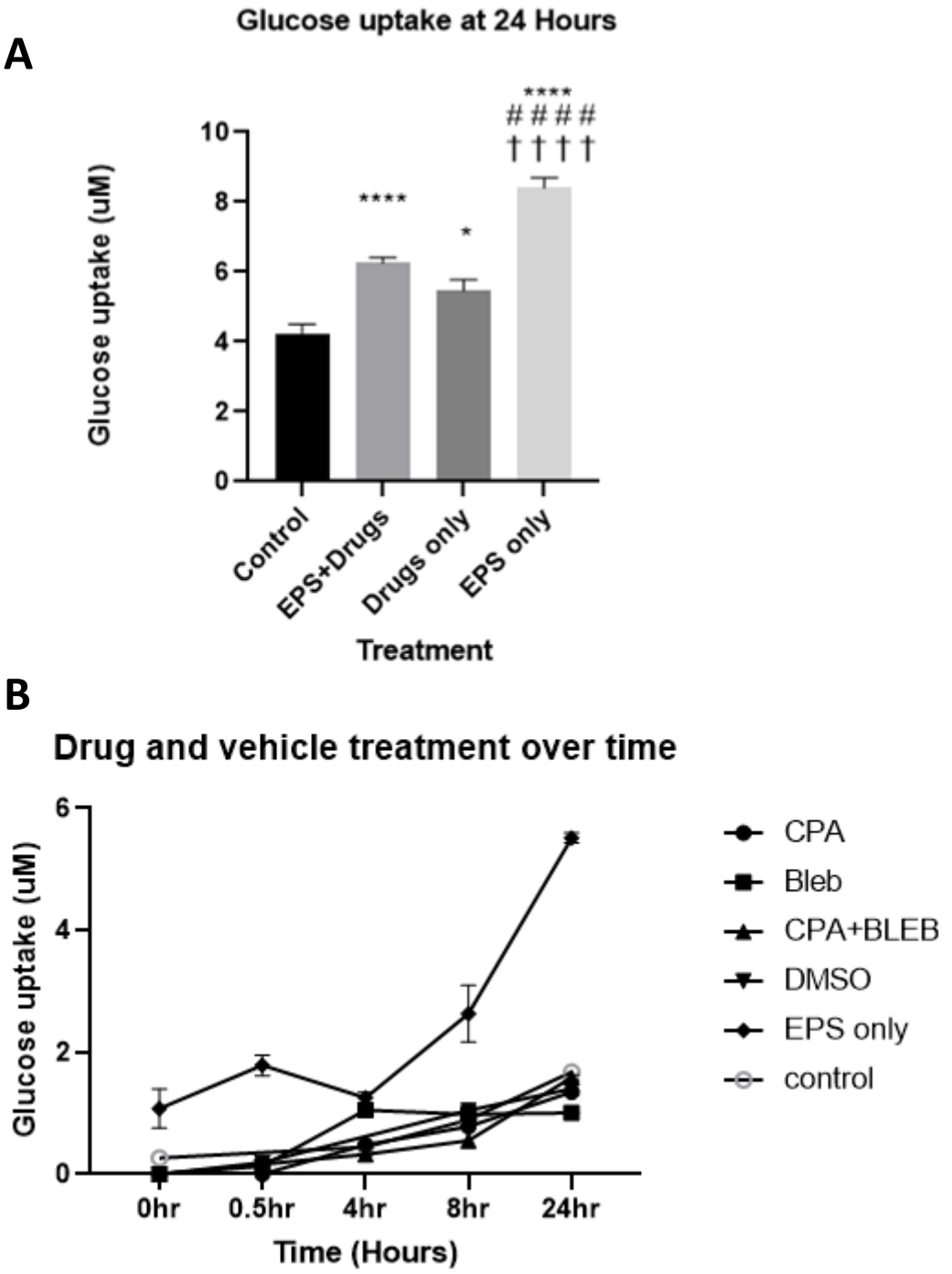


Figure 4.10: glucose uptake as measured by 2-DG accumulation at 24 hours (A) and over 24 hours (B) with continuous EPS. Error bars indicate SEM. N=3.

Individually in the absence of EPS, blebbistatin and CPA had no effect on glucose uptake over 24 hours. This matched the DMSO vehicle control. EPS had a strong effect on glucose uptake, with significance becoming apparent at 8 hours and increasing at 24 hours ($p= 0.0149$ and <0.0001). In combination the drugs had a small significant effect on glucose uptake (0.0137). When EPS was applied in addition to both CPA and blebbistatin the increase in uptake was slightly larger, more significant (<0.0001) (**Figure 4.10)**. This increase was still smaller that the increase elicited by EPS alone however (6.25uM vs 8.40uM, with a p value of <0.0001).

4.3 Discussion

Treatment of cells with blebbistatin alone, Cyclopiazonic acid alone or in combination for 24 hours resulted in changes to anabolic signalling, calcium responsive biomarkers and glucose uptake in line with what would be expected to correspond with a disuse state.

The lack of change in glucose over time with either CPA or blebbistatin in the absence of EPS could be due to growth and maintenance of the cells in high (4.5g/L) glucose media, which contains much more glucose than cells would be exposed to physiologically (0.7-0.9g/L) (Triplitt, 2012). This was necessary in order to achieve rapid growth as well as to prevent depletion of glucose during EPS, which is known to occur after approximately 18hr of culture in low glucose media (1g/L) (Farmawati *et al*, 2013). Under these high glucose conditions, it may not be necessary for contracting

cells to utilize glycogen stores, as oxidizable substrate is readily available in the environment (Hamada *et al*, 2003). Many studies have demonstrated depletion of glycogen stores in c2c12 in response to EPS (Manabe *et al*, 2012; Marotta *et al*, 2018; Li *et al*, 2018), which might be explained by differences in voltage or pulse duration between these studies and our own.

Media lactate behaved much more conventionally, with EPS producing a marked increase in media lactate, which was not seen with any of the individual or combined drug treatments. The magnitude of increase was consistent with the findings of other groups using the same EPS protocol for the same duration with high glucose media, approximately a 3-fold increase in lactate (Lautaoja *et al*, 2021).

4.3.1 Signalling changes in response to CPA and Blebbistatin alone and in combination.

Cells treated with only DMSO as a vehicle control showed a decrease in viability as well as an increase in mTOR phosphorylation over time, which was matched by P70 (T389) phosphorylation increase. This was taken as a sign that suspension of both CPA and blebbistatin in 100% DMSO resulted in excessively high DMSO concentration (approximately 1.15% v/v) and so blebbistatin was instead suspended in 27% DMSO for future experiments. This resulted in a lower (0.44% v/v) concentration of DMSO whilst still solubilizing the required blebbistatin mass.

As expected, treatment of cells with blebbistatin alone did not induce changes in anabolic signalling. Similarly, EEF2 (T56) phosphorylation did not change, interpreted here as a lack of change in calcium signalling, again, in line with expectation. This

assumption was made because EEF2 phosphorylation is controlled by a single kinase- EEF2K- which is considered dependent on calmodulin (Hizli et al, 2013). Indeed, changes in calmodulin that regulate EEF2 are associated with suppression of protein synthesis during exercise, which was not dependent on AMPK activation (Rose *et al*, 2009). Therefore, EEF2 phosphorylation can be used as an indirect measurement of cytosolic calcium. The only significant change over time with treatment with blebbistatin was in ERK1/2, which declined from 8 hours onwards. As it pertains to cellular survival (Lu and Xu, 2006), a decrease in phosphorylation of ERK1/2 is generally a pro-apoptotic change, though not always. Though this could reflect changes in survival signalling owing to reduction in cell movement, the fact that this change is also seen with CPA treatment alone suggests that this is not the case.

Treatment with CPA alone coincided with more significant changes in cell signalling as might be expected given the importance of calcium flux in normal function of skeletal muscle (Hyatt and Powers, 2020; Shur *et al*, 2022). The large increase in EEF2 phosphorylation between 8 and 24 hours of CPA treatment is a good indication of an increase in cytosolic calcium ion concentration. CPA has been used in other studies (Sekiguchi *et al*, 1999) to invoke contraction on an acute timescale. The switch from low to high EEF2 phosphorylation roughly matches the switch from high to low P70 phosphorylation. If the two were related it could be indicative of a reduction in protein synthesis brought about by dysregulated calcium signalling, as seen in rodents (Lewis *et al*, 1982) and strongly suspected in humans (Agrawal *et al*, 2018; Parry and Puthucheary, 2015). The increase in 4EBP1 (T37/47) phosphorylation at 4 hours when

compared to the 24-hour timepoint could be seen as contradictory to reduction in protein synthesis due to elevated calcium, however, this could be explained by an increase related to autophagy (Tsai *et al*, 2015). Calcium responsive mechanisms involved In modulation of skeletal muscle protein synthesis, but independent of 4EBP1 activity (Ferreira *et al*, 2019) have been demonstrated in mice previously.

Whilst the purpose of many signalling proteins is understood in isolation, exactly how they function in cross tissue interactions and in the establishment of stable feedback loops within skeletal muscle under dynamic conditions is not well understood. We found that by applying both CPA and blebbistatin for 24 hours phosphorylation of P70 at Thr-389 decreased significantly, with and without EPS. Additionally, a matching increase was seen in 4EBP1 phosphorylation at Thr37/46. Whilst no significant change was observed in mTOR phosphorylation, it was clearly seen that EPS alone depressed mTOR phosphorylation below the basal level, which was also seen with 4EBP1. This could be interpreted as exercise induced suppression of anabolic signalling, given that cells were harvested immediately after finishing the 24-hour EPS program. Suppression of P70 and 4EBP1 during exercise has been reported previously (Atherton and Smith, 2012; Dreyer *et al*, 2006), with most suppression being seen to occur in resistance exercise rather than endurance exercise. Despite this, a sufficient volume of work could deplete ATP to the point of inhibition of mTOR via AMPK activation (Knuiman *et al*, 2015). In humans this is generally accompanied by depletion of glycogen, which we did not observe. Treatment with CPA and blebbistatin (CB) as well as CPA and blebbistatin with EPS produced the same change in P70 and 4EBP1 expression (decrease and

increase respectively), which was expected, as blebbistatin prevented contraction from occurring as a result of depolarization caused by EPS. The increase in 4EBP1 phosphorylation seen with CB indicates increasing rates of translation initiation, which occurs when 4EBP1 becomes unbound from eIF4E as a result of phosphorylation (Qin *et al*, 2016).

Many health conditions and behaviours lead to loss of muscle mass, including bedrest and immobilization. Both of these coincide with development of insulin resistance (Stuart *et al*, 1988; Alibegovic *et al*, 2009) however, despite the shared outcome of reduction in muscle mass, the signalling events leading to this can be quite different (Greenhaff *et al*, 2019). It is generally accepted that as the primary controller of muscle protein synthesis, mTOR phosphorylation and by extension P70 and 4EBP1 phosphorylation should decrease during muscle wasting (Gao *et al*, 2018). However, human immobilization studies do not consistently show this, instead showing reduction in muscle mass, without change in mTOR, 4EBP1 or P70 Phosphorylation, instead seeming to rely on autophagic processes (Møller, *et al*, 2019). This suggests either that multiple mechanisms work alongside the traditionally considered mTOR pathway to facilitate muscle loss during unloading or time sensitive changes in signals that feed back into their own regulation. A similar discrepancy can be seen in rodents whereby the processes involved in muscle wasting appear to differ between HLS (hindlimb suspension) and casting, given that the mass of muscle lost in the cast limb is significantly more than the suspended limb, yet protein synthesis, P70 and 4EBP1 phosphorylation are also significantly higher (Speacht *et al*, 2018). Rodent studies have

shown that in early immobilization- up to 3 days- mTOR activity is increased, after which activity is reduced or equivalent to baseline (You *et al*, 2015). Importantly, the early increase in mTOR phosphorylation has a negative effect on maintenance of muscle mass, thought to be caused by reduction in ribosomal biogenesis (Rozhkov *et al*, 2022; Leermakers *et al*¸2019). This would explain the lowering of P70 phosphorylation whilst 4EBP1 rises in response to CB treatment.

EEF2 phosphorylation significantly increased in response to EPS alone and drugs alone but not EPS and Drugs in combination. This would suggest that the increase in calcium concentration in response to EPS is greater than in response to CPA or CPA and blebbistatin, according to our results. Presumably, the difference in outcomes can be attributed to EPS inducing a transient release of calcium, whilst CPA application leads to sustained elevation of calcium due to the prevention of reuptake by SERCA pumps (Wakizaka *et al*, 2017).

Protein Kinase B phosphorylation (T308) only changed slightly from control in response to CB applied alongside EPS. PKB phosphorylation is known to play a role in GLUT 4 recruitment, however, this is primarily as part of the insulin responsive pathway (Hajduch *et al*, 2001), which we did not directly manipulate here. The likely explanation for the change is suppression which becomes greater the longer contraction persists, which is supported by the presence of an initial spike in PKB phosphorylation at 0.5 and 4 hours, followed by return to baseline levels at 8 and 24 hours in cells treated with DMSO or CB (100%).

4.3.2 Glucose uptake in response to drugs

Treatments which did not include EPS did not alter glucose uptake significantly from untreated control values. As was expected and in line with literature (Nikolić *et al*, 2012), EPS of myotubes increased glucose uptake approximately threefold. For all groups a significant increase in glucose can been seen with increasing time, which for the most part can be explained as constitutive GLUT1 activity in a high glucose environment. In the case of CPA only, a calcium derived increase in glucose uptake was expected to occur as a result of calcium mediated glucose uptake (Youn *et al*, 1991, but this was not apparent in our timepoint experiment. An explanation for this could be that 24 hours is a long enough time for elevated calcium to cease its function as an acute stimulator of glucose uptake and begin processes which inhibit glucose uptake chronically (Uryashi *et al*, 2022). Glucose uptake over time shown in **figure 4.10B** suggests that rather than reducing glucose uptake CPA and blebbistatin – in combination or alone- prevent the increase in glucose uptake that contraction would otherwise bring about. This would not impede calcium dependent glucose uptake (Saito *et al*, 2016), which is likely the cause of the increased glucose uptake shown in **figure 4.10A** for cells treated only with drugs. Multiple studies have shown that calcium release, even at levels too low to induce contraction (Youn *et al*, 1991; Röckl *et al*, 2008) are capable of stimulating glucose uptake. The mechanism for this has not been fully explored, though CamKII- AMPK interaction is proposed (Witczak *et al*, 2010) as is crossover with the insulin dependent glucose uptake pathway (Kjøbsted *et al*, 2019). However, the slight rise in glucose uptake above untreated control values (shown in

figure 4.10A) suggests that a calcium mediated increase in glucose uptake via CPA activity may still be possible at 24 hours.

4.3.3 qPCR Biomarker response

Expression of mRNA transcripts for CALM1 and ATP2A1 (encoding calmodulin 1 and SERCA1 respectively) were seen to decrease considerably compared to untreated controls in response to EPS, to undetectable levels for CALM1. Conversely, expression increased with drug treatment, both with and without EPS. Several studies have shown expression of these transcripts increases in response to bedrest (Shur *et al*, 2022; Chopard *et al*, 2009). Deruisseau *et al* (2005) observed that in diaphragm muscle, calmodulins 1 and 2 undergo opposing regulation, with CALM1 increasing during atrophy and CALM2 decreasing and speculated that this may be related to sub-cellular localisation of signalling changes requiring calmodulin.

Changes in ATP2A1 transcript expression are less well studied, however it is known that mutation in the gene can lead to muscular disease characterised by calcium handling irregularities such as Brody myopathy (Guglielmi *et al*, 2013). Elevation in these two biomarkers as well as in EEF2 phosphorylation in response to combined CPA and blebbistatin treatment was taken as an indicator that a state of persistently elevated cytosolic calcium had been achieved. It was hoped that by recreating the calcium dysregulated environment that is found *in vivo,* the required signalling patterns for impaired glucose uptake would also be established. The fact that glucose uptake did not drop below the level seen in untreated cells suggests that glucose uptake had been

prevented but not necessarily impaired. It is possible that glucose uptake was in fact impaired, but that the difference between impaired and basal uptake was not pronounced enough to be apparent at 24 hours.

4.4 Limitations section

One of the main concerns with using CPA to manipulate cytosolic calcium concentration was that it might become difficult to differentiate an effect that is indicative of acute contraction from one which is indicative of a developing disuse phenotype. In order to minimize this issue, blebbistatin was applied before CPA in all experiments where the two were used together in order to prevent contraction as a result of calcium release.

Use of blebbistatin to prevent contraction came with the possible drawback of the potential for phototoxicity, in which visible light wavelengths, particularly blue light, causes rapid degradation of blebbistatin. Cytotoxicity is thought to be a function of the intermediates produced during breakdown, rather than persistent products (Kolega *et al*, 2004), the production of which does not stop once the drug is returned to darkness. Viability was assessed by resazurin to resorufin conversion (not shown) and it was determined that phototoxic products were not being produced. The second issue is that both CPA and blebbistatin were suspended in DMSO, which can have a number of effects on cells depending on the concentration. At high concentrations (>5%) DMSO Exhibits noticeable effects on contractile elements of skeletal muscle (Mariano *et al*, 2001), whilst at lower doses the effects can be more subtle, but still potentially

experiment confounding (Orzechowski *et al*, 2001). We ruled out excessive effects of DMSO based on a lack of change in EEF2 phosphorylation once blebbistatin was suspended in a 27% solution as well as no change in cell viability.

Another potential limitation of this study is the use of biomarkers to indirectly ascertain the calcium ion concentration in the cytoplasm. This method was chosen over more precise means such as calcium probes because excitation wavelengths often fell within the band that would cause phototoxicity due to breakdown of blebbistatin, as well as requiring additional microscopy (Russell 2011; Bkaily *et al*, 2017).

Conclusion

After adjusting the concentrations of drugs to reach a sustainable final concentration of DMSO we were able to achieve a treatment of CPA and blebbistatin which did not impair cell viability. Individually, CPA was able to elevate cytosolic calcium and blebbistatin was able to prevent contraction as we had hypothesized. By applying blebbistatin and CPA together we were able to prevent glucose uptake from being stimulated by EPS. This was accompanied by a matched decrease in lactate output, suggesting that the EPS induced increase in glycolysis had been entirely prevented We were also able to induce changes in anabolic signalling that is consistent with what we have come to expect from muscle disuse, with reduction in phosphorylation of P70 as well as ERK1/2. Judging by changes in EEF2 phosphorylation as well as the significant increase in CALM1, CPA was able to increase the cytosolic concentration of calcium as we had predicted, though direct measurement of calcium would be needed to confirm this. These endpoints suggest that the CPA+ blebbistatin treatment induced changes that were

comparable to what is seen in muscle disuse and that this could be used as a model of disuse with some refinement.

Chapter 5: Attempted treatment of the CB model with AICAR and Dantrolene in conjunction with EPS

5.0 Introduction

In the previous chapter we demonstrated that application of blebbistatin and CPA could prevent contraction in response to 24 hours of continuous EPS and induce changes in biomarkers and protein EEF2 phosphorylation that is indicative of persistently elevated cytosolic calcium. We observed that these changes were paired with a reduction in glucose uptake, lactate output and reduction in P70 (T389) phosphorylation when compared to cells that only experienced EPS. Prevention of decrease in 4EBP1 (TT37/46) and mTOR (S2448) phosphorylation as well as increase in ERK1/2 (S217/221) phosphorylation associated with EPS was also recorded. Following on from this, we were interested to know if molecular manipulation intended to prevent elevation of cytosolic calcium or stimulate AMPK could reverse changes initiated by CPA and blebbistatin.

Dantrolene is a muscle relaxant used to treat malignant hyperthermia- a condition that most commonly occurs as an adverse reaction to anaesthetics (Glahn *et al*, 2020). It binds the ryanodine receptor and stabilizes the channel in the closed position, preventing the passage of Ca^{2+} to the cytoplasm (Kobayashi *et al,* 2005). In a previous study (Park *et al*, 2013), it was shown that dantrolene can be used to prevent calcium release elicited by caffeine application to C2C12 cells. Our own model elevates calcium not by enhancing release with caffeine, but by preventing re-uptake from the cytosol. By preventing initial release to the cytosol from occurring, we hope to prevent the establishment of abnormal calcium signalling, negating the effect of inhibition of SERCA caused by CPA. Whilst contraction from EPS will cause an increase in cytosolic calcium, this will be transient. It also will not occur in the presence of CPA alone, as CPA will cause a sustained release of calcium, depleting the SR calcium store and producing a

sustained contraction. Furthermore, this sustained CPA induced contraction will not be present when blebbistatin is also present, leaving only elevated cytosolic calcium.

In the previous chapter, blebbistatin was applied in order to prevent contraction from occurring in response to EPS. Though this is an irreversible process, some of the downstream signalling that leads to contraction mediated glucose uptake can likely be restored by application of AICAR. As this acts downstream of excitation-contraction coupling, the remainder of the pathway should be exploitable, allowing GLUT4 recruitment and subsequent glucose uptake to continue unimpeded. AICAR is a molecular mimic of AMP and as such can activate AMPK, the cellular energy sensor (Kim *et al*, 2016), which is partly, but not wholly responsible for contraction mediated glucose uptake. Activation of AMPK is known to associate with phosphorylation and subsequent activation of TBC1D1 and TBC1D4, which are implicated in both insulin stimulated and contraction stimulated GLUT4 vesicle trafficking through interaction with Rab proteins which coat the GLUT4 bearing vesicles (Cartee, 2015; O'Neill, 2013). It is the AMPK-TBC1D signalling pathway which we expect remains intact downstream from the blebbistatin impaired contractile apparatus The remaining fraction can be attributed to mechanical stimulation that occurs during contraction, but which can also be reproduced by stretching muscle *ex vivo* (Jensen *et al*, 2014).

5.01 Aims and hypothesis

The aim of this set of experiments was to determine the ability of the drugs AICAR, dantrolene, as well as an AICAR and dantrolene combination to restore glucose uptake in cells treated with CPA and blebbistatin. Additionally, we wished to know if EPS could improve the ability of these drugs to restore glucose uptake.

5.1 Methods

5.1.1 Cell culture

Cell culture of C2C12 cells was performed as described in chapter 2. Cells were seeded such that all of the cells used were between days 5 and 6 post-differentiation at the time of harvesting. Vials containing cells between passages 9 and 11 were used. For the purposes of this chapter, n=3 refers to 3 wells of a plate.

5.1.2 Drug treatments

Three groupings of drug treatments were performed in order to examine both the response of cells to the treatment as well as the potential mechanism by which the reaction was induced. Firstly, to ascertain the effect of the drugs with contraction, cells were treated with dantrolene (D) (D9175) or AICAR (A) (A9978) or with both AICAR and dantrolene (AD), with EPS (**Figure 5.1**). All of the drugs were purchased from Sigma. Dantrolene was dissolved with 187 mg/ml mannitol, however no additional vehicle control was run as mannitol is present in commercial formulations of dantrolene to allow physiologically useful concentrations of dantrolene to be achieved without DMSO (Krause *et al*, 2004). EPS was performed as described previously at 11.5V, 2ms, 1 Hz for 24 hours.

Secondly, to determine the interaction of CPA and Blebbistatin when applied individually with dantrolene, AICAR or AD these drugs were applied and incubated for 24 hours to compare the reaction between each combination. EPS was applied to all treatment combinations in this experiment. Cells were harvested after 24 hours of treatment.

Lastly, in order to determine the effect of EPS on the interaction of drugs in the absence of interference by CPA or blebbistatin, cells were split into two groups, those receiving a rescue drug with no EPS and those receiving a rescue drug alongside EPS. Cells were harvested after 24 hours of treatment.

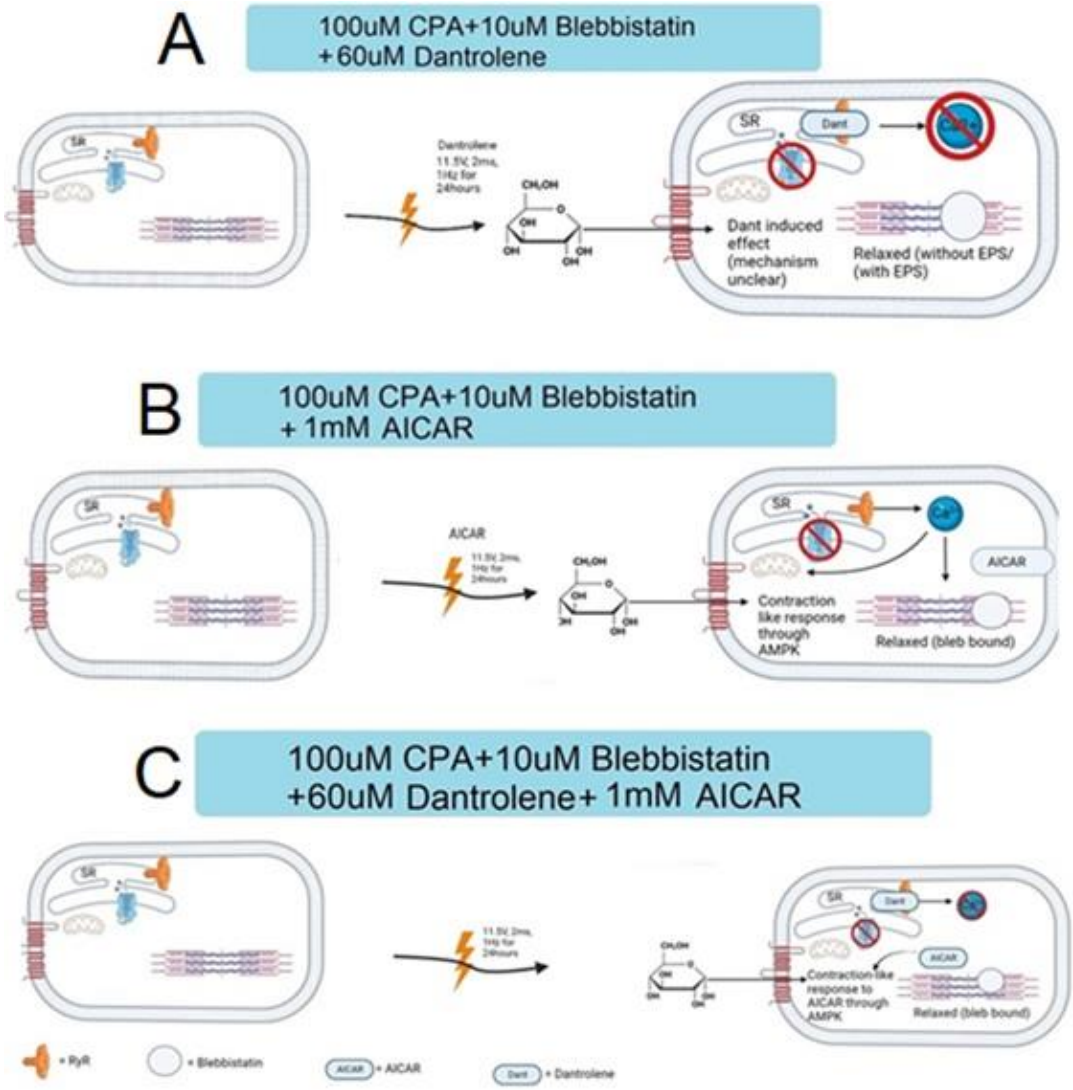

A
100uM CPA+10uM Blebbistatin
+60uM Dantrolene
SR
Dant
Dantrolene
11.5V, 2ms,
1Hz for
24hours
Dant induced
effect
(mechanism
unclear)
Relaxed (without EPS/
(with EPS)
B
100uM CPA+10uM Blebbistatin
+ 1mM AICAR
AICAR
Contraction
like response
through
AMPK
Relaxed (bleb bound)
C
100uM CPA+10uM Blebbistatin
+60uM Dantrolene+ 1mM AICAR
Contraction-like
response to
AICAR through
AMPK
Relaxed (bleb bound)
= RyR
= Blebbistatin
= AICAR
= Dantrolene

Figure 5.1: Diagrammatic depiction of the major treatment combinations performed in this chapter and the expected outcomes from each intervention.

Dantrolene (A) is a muscle relaxant that works by stabilising the RyR pore in the closed position, preventing Ca^{2+}ions from entering the cytoplasm. This is intended to counteract the effect of CPA, which was applied to induce elevated cytosolic Ca^{2+} by preventing reuptake by SERCA2 ion pumps.

AICAR (B) was chosen to stimulate the contraction related glucose uptake that remained viable after treatment with blebbistatin prevented contraction. This is achieved by mimicry of AMP, which causes activation of AMPK via phosphorylation of T172. AMPK, alongside cytosolic Ca^{2+} / calmodulin is responsible for regulation of the phosphorylation of TBC1D1 and TBC1D4, involved in GLUT4 vesicle trafficking.

5.1.3 EPS protocol

EPS was performed continuously as described previously. Pulse train settings were maintained at 11.5V, 2ms, 1Hz as described in chapter 2, with a 1-hour pre-stimulation period to induce contractile sarcomere formation. Drugs were applied immediately after pre-stimulation and before 24 hours of contraction was applied.

5.1.4 Lactate assay

Measurement of media lactate concentration was performed as described in chapter 2

5.1.5 Glycogen assay

Measurement of cell glycogen was performed as described in chapter 2

5.1.6 Glucose uptake

Measurement of glucose uptake as internal 2DG6P was performed as described in chapter 2, with slight modification. Rather than performing 1 injection of 6 separate samples, 3 samples were used for each timepoint, injected twice. This modification was made to reduce mass spectrometer running time and cell culture requirements.

5.1.7 Measurement of signalling

Anabolic signalling was measured by western blotting as described in chapter 2. Additionally, targets EEF2, ERK1/2 and PKB (Akt) were measured.

5.1.8 Data analysis

Data analysis was performed as described in chapter 2.

5.2 Results

5.2.1 Dantrolene or AICAR do not restore glucose uptake in the disuse model when paired with EPS.

The disuse model created in the previous chapter- referred to as the treatment "CB" in figures- was subjected to combinations of the drugs AICAR and Dantrolene in conjunction with EPS in order to determine the most effective method to restore normal glucose metabolism.

5.2.2 Dantrolene

The disuse model reduced glucose uptake from 8.4uM to 6.4uM ($p=0.0002$), but treatment of the model with dantrolene in addition to EPS further decreased glucose uptake down to 4.3uM ($p<0.0001$) over a 24-hour period. However, this further decrease in glucose uptake was not reflected in lactate output, which was restored to control levels by dantrolene + EPS treatment. No significant change in glycogen concentration compared to the EPS receiving control group

was observed as a result of the disuse model or treatment of that model by dantrolene (**Figure 5.2**).

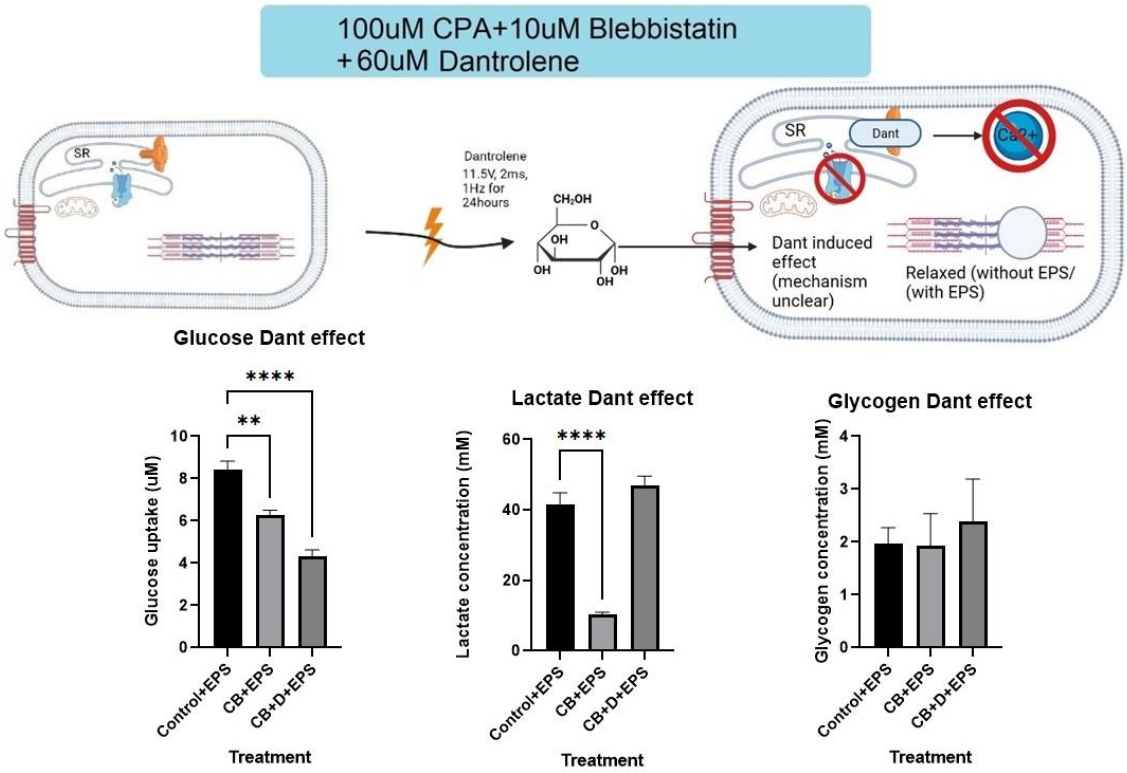


Figure 5.2: Diagram of expected changes occurring during experimental drug treatment. The C2C12 disuse model was treated with EPS and 60µM dantrolene for 24 hours before harvesting. CB= CPA+ Blebbistatin. Data are presented as means. Error bars indicate SEM. N=3 (Glucose and Glycogen). N=6 (Lactate). ****indicates p<0.0001

The restoration of lactate output was associated with a significant (p= 0.0193) reduction in 4EBP1 Phosphorylation, back to the level seen in unstimulated c2c12 cells (**Figure 5.3**). Changes in P70 phosphorylation (T389) and mTOR (S2448) from EPS alone were not found to be statistically significant. A large and significant (p<0.0001) increase in ERK1/2 phosphorylation was also observed, increasing 5.7 times above the basal level, which was not seen with EPS alone. EEF2 phosphorylation at Threonine 56 decreased to near undetectable

levels with Dantrolene and EPS treatment (p=0.0033). Change in p-PKB (T308) was minimal and not statistically significant, with the Dantrolene+ EPS treatment having the highest variability of the 4 treatments.

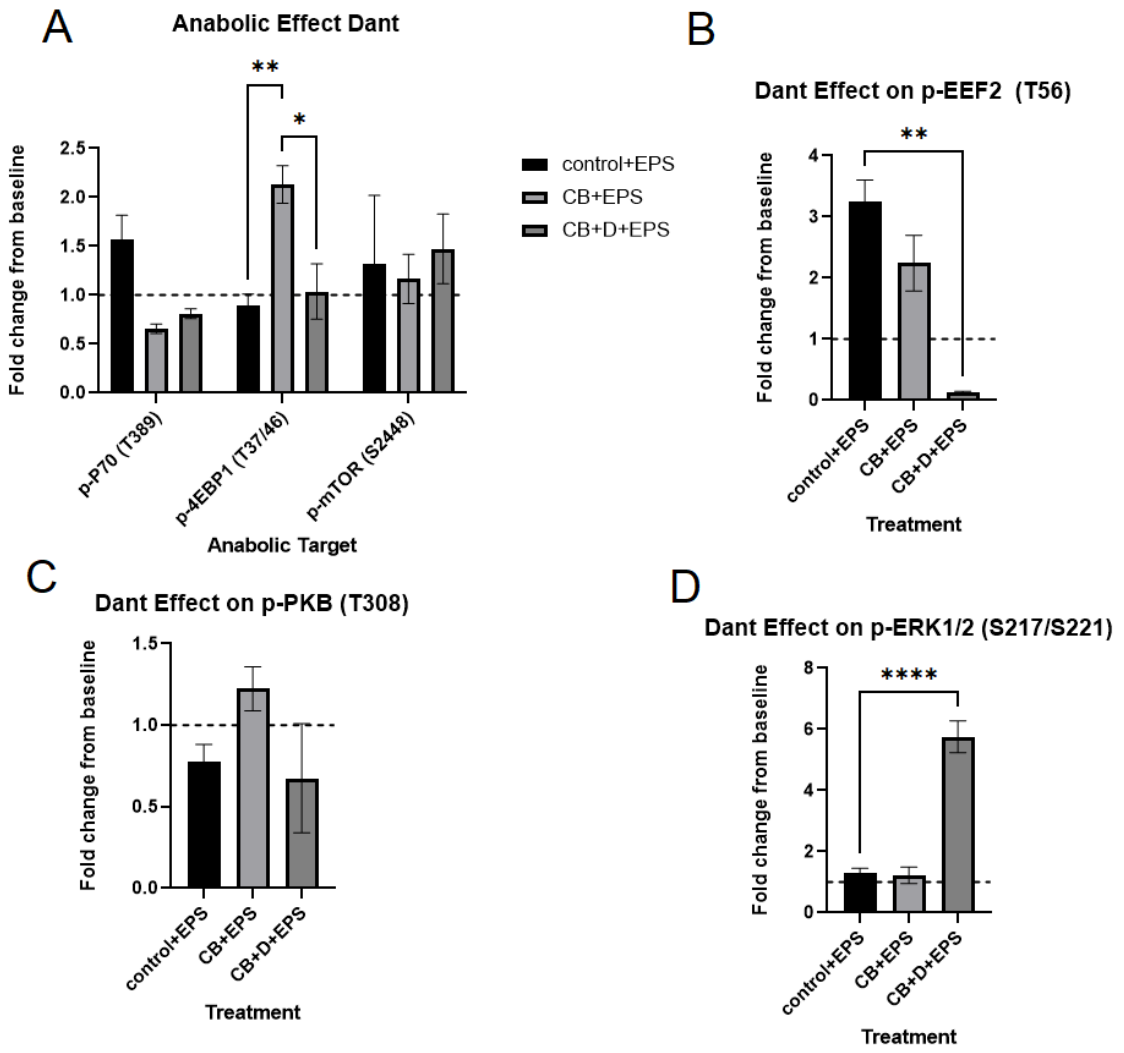


Figure 5.3: Changes in anabolic signalling and EEF2, PKB and ERK1/2 phosphorylation and accompanying representative western blots. Cells were treated with either EPS alone, CPA and blebbistatin (CB) with EPS or CB and dantrolene with EPS for 24 hours and then harvested. Error bars indicate SEM. N=6 or 3 as indicated by representative western. Dashed line indicates baseline phosphorylation found in cells at the same timepoint that received no treatment or EPS.

5.2.3 AICAR

Treatment of the disuse model with AICAR alone (**Figure 5.4**) caused glucose uptake to decrease even further from the EPS treated control than CB treatment alone ($p<0.0001$). Lactate output was elevated back towards normal values for EPS treated cells (dantrolene= 47mM, AICAR= 24mM, AD= 27mM compared to BC = 10mM.) AICAR treated lactate output remained significantly below EPS treated control values ($p<0.0001$). AICAR treatment did not produce a change in glycogen concentration.

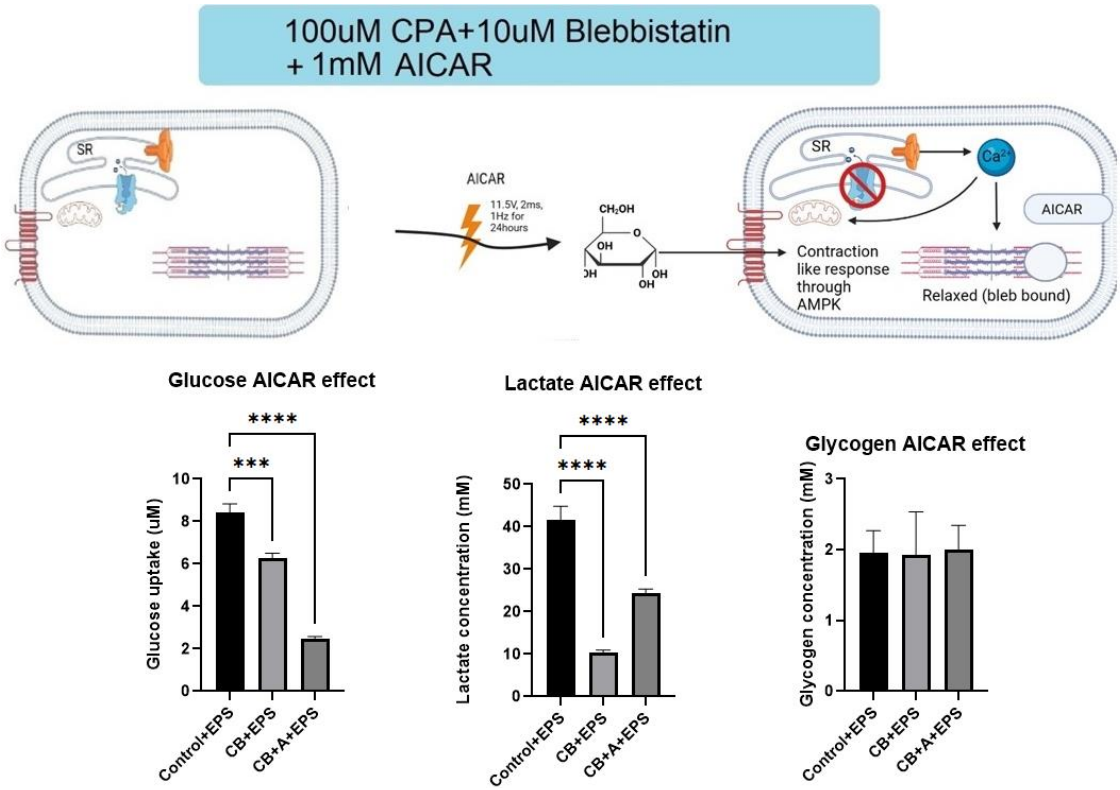


Figure 5.4: Diagram of expected changes occurring during experimental drug treatment. The C2C12 disuse model was treated with EPS and 1mM AICAR for 24 hours before harvesting. CB = CPA + Blebbistatin. Data are presented as means. Error Bars indicate SEM. N= 3 (Glucose and Glycogen). N=6 (Lactate). **** indicates $p<0.0001$. *** indicates $p= 0.0001$.

Treatment with AICAR+EPS significantly (p= 0.0056) reduced P70 phosphorylation at T389, depressing it even further than the treatment with CB (**Figure 5.5)**. There was no change in 4EBP1 Phosphorylation at T37/T46 and mTOR phosphorylation at S2448. No change was observed in p-EEF2 (T56) and p-PKB (T-308) and ERK1/2 at S217/S221.

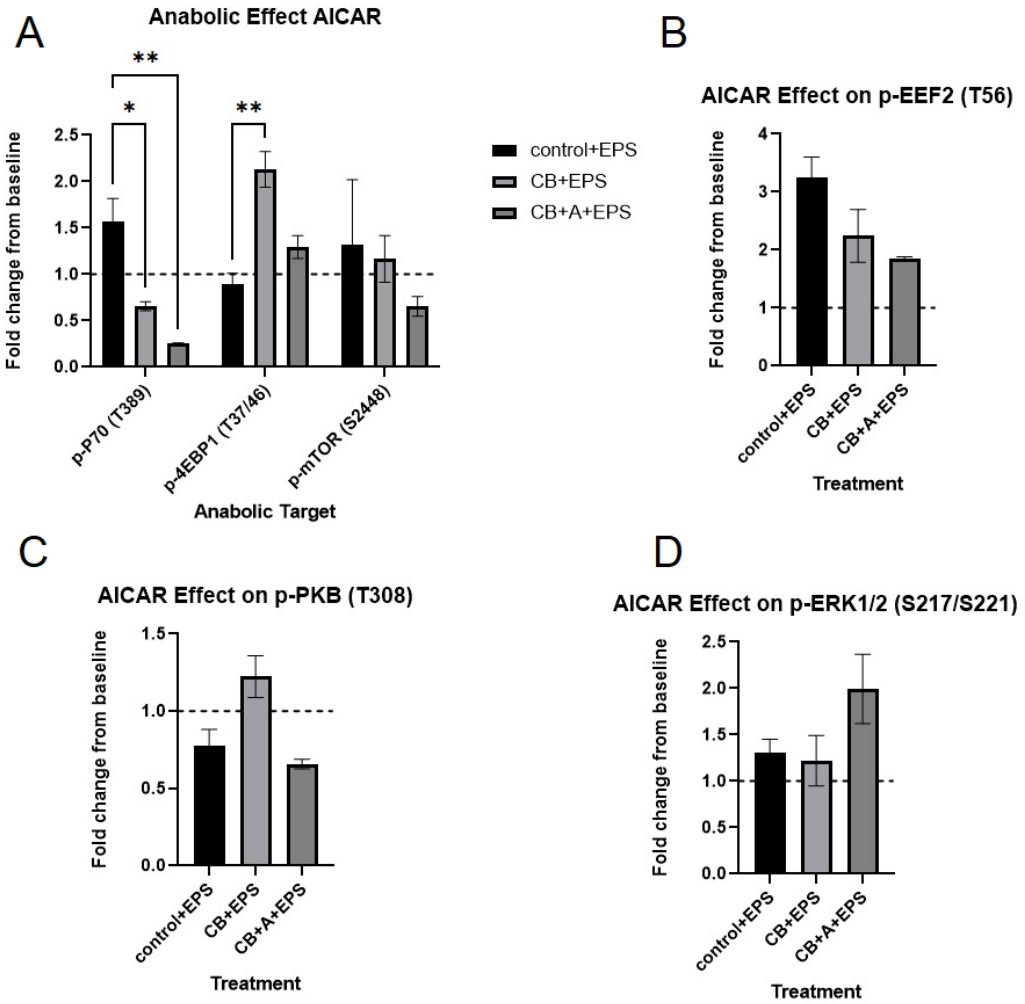


Figure 5.5: Changes in anabolic signalling and EEF2, PKB and ERK1/2 phosphorylation and accompanying representative western blots. Cells were treated with either EPS alone, CPA and blebbistatin with EPS (CB) or CB and AICAR with EPS for 24 hours and then harvested. Error bars indicate SEM. N=6 or 3 as indicated by representative western. Dashed line indicates baseline phosphorylation found in cells at the same timepoint that received no treatment or EPS.

5.2.5 AICAR+dant

A combination of dantrolene and AICAR, also failed to restore glucose uptake to the levels seen with EPS alone ($p<0.0001$). Combined AD treatment **(Figure 5.6)** lactate output remained significantly below EPS treated control values ($p= 0.0370$). A decrease from 1.9mM to 1.5mM glycogen was seen with AD treatment though this was -again- not significant statistically.

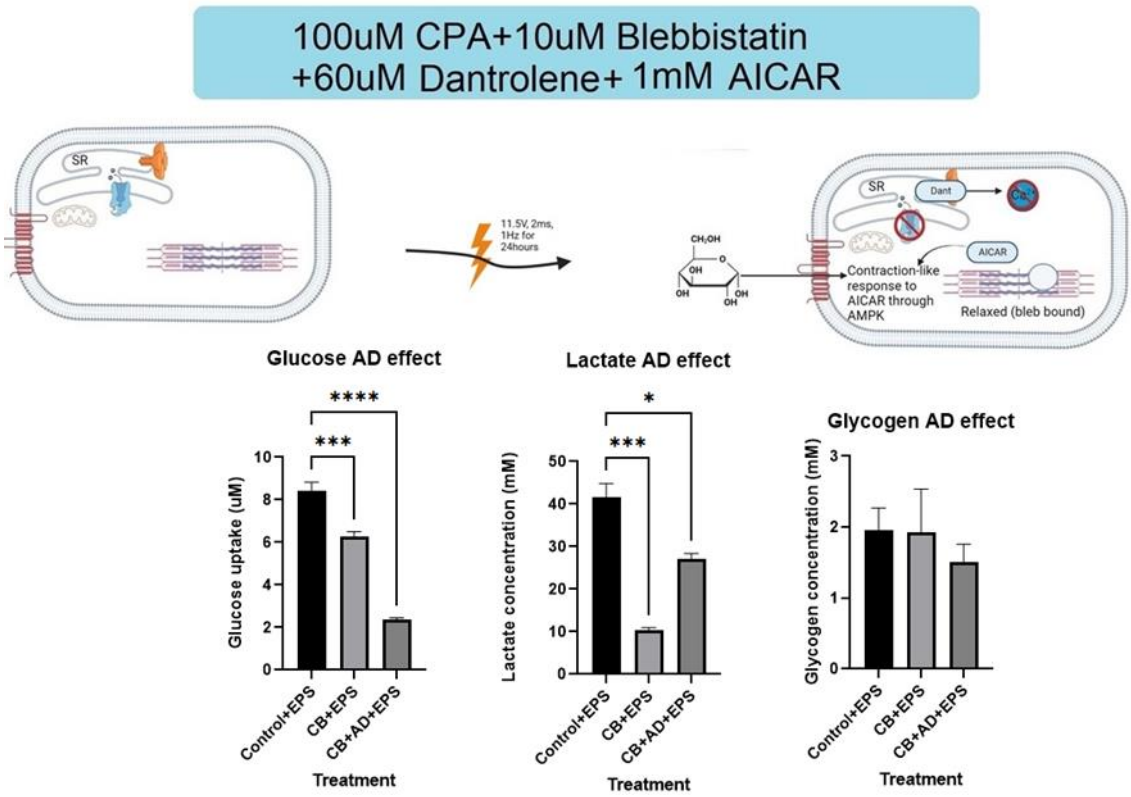


Figure 5.6: Diagram of expected changes occurring during experimental drug treatment. The C2C12 disuse model was treated with EPS and 60µM Dantrolene+ 1mM AICAR for 24 hours before harvesting. CB = CPA + Blebbistatin. Data are presented as means. Error Bars indicate SEM. N= 3 (Glucose and Glycogen). N=6 (Lactate). **** indicates p<0.0001. *** indicates p value between 0.0001 an 0.001.

Treatment with a combination of 1mM AICAR and 60µM Dantrolene as well as EPS depressed both P70 and 4EBP1 phosphorylation when compared to EPS control (**Figure 5.7)**. P70 phosphorylation decreased to levels significantly less than the EPS control (p=0.0058)-from 1.6 to 0.2-fold baseline-whilst the depression of 4EBP1 phosphorylation was not significantly different from the EPS control. Conversely, though lower than phosphorylation during CB induced disuse, P70 did not significantly differ during AD treatment, whilst 4EBP1

phosphorylation was significantly lower as a result of AD treatment than it was during CB induced disuse ($p<0.0001$). Though it was not significant, there was a matching decrease in mTOR phosphorylation, approximately the same as seen with dantrolene+EPS treatment. Interestingly, although AICAR+EPS and AICAR+dantrolene+EPS show the same pattern of effects on mTOR and P70 phosphorylation, this is not the case for 4EBP1. Despite dantrolene+EPS and AICAR+EPS having a similar and small effect on 4EBP1 phosphorylation, AICAR and Dantrolene together with EPS massively reduced 4EBP1 phosphorylation.

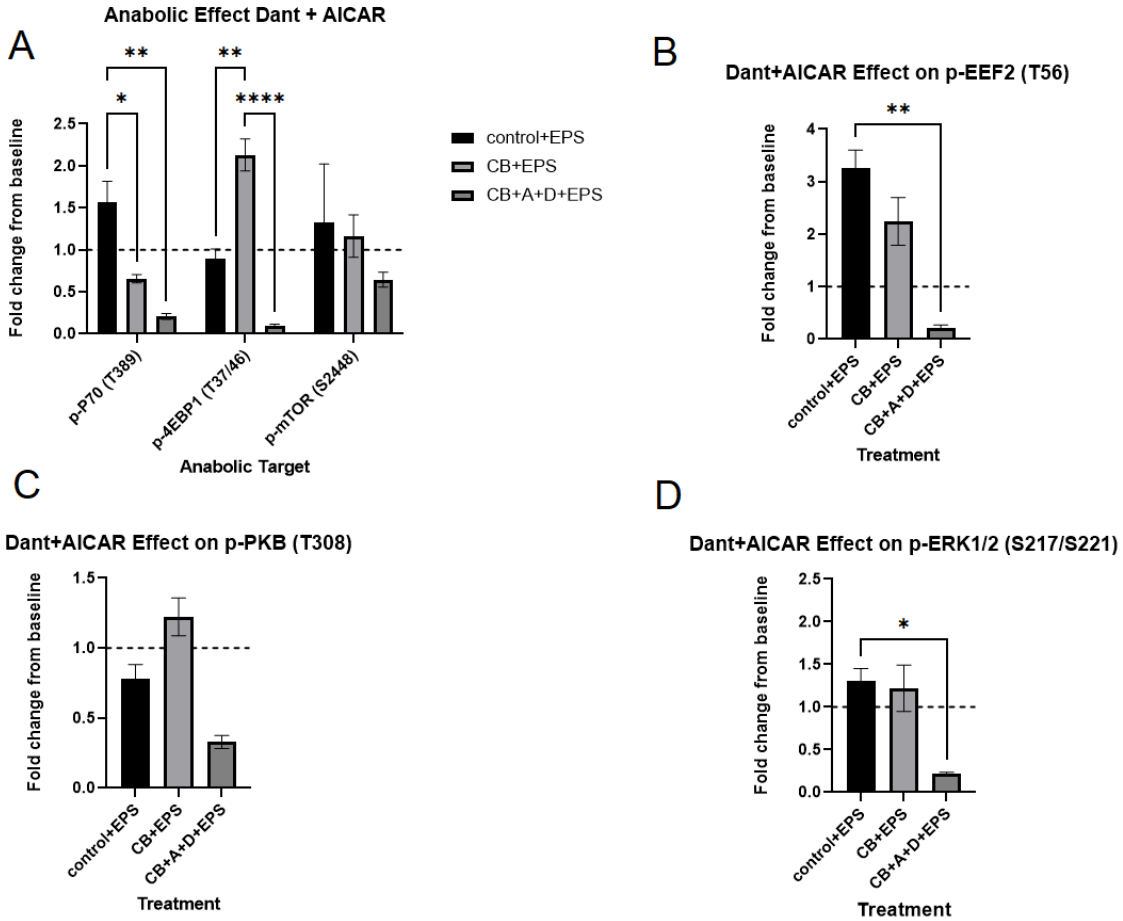


Figure 5.7: Changes in anabolic signalling and EEF2, PKB and ERK1/2 phosphorylation and accompanying representative western blots. Cells were treated with either EPS alone, CPA and blebbistatin with EPS (CB) or CB and 1mM AICAR+ 60µM dantrolene with EPS for 24 hours and then harvested. Error bars indicate SEM. N=6 or 3 as indicated by representative western. Dashed line indicates baseline phosphorylation found in cells at the same timepoint that received no treatment or EPS.

As with dantrolene+ EPS, dantrolene+AICAR heavily reduced phosphorylation of EEF, resulting in a significant difference from EPS control (p= 0.0040), though not from CB induced disuse. Despite a moderately large decrease in phosphorylation of PKB, the difference between dantrolene+ AICAR+ EPS treatment and EPS control as well as CB induced disuse was not significant. Unlike any of the other drugs applied alone, there was a weakly significant decrease in ERK1/2 phosphorylation when compared to the EPS control (p=0.0481).

5.2.7 EPS does not improve the ability of selected drugs to increase glucose uptake in normal baseline cells.

In order to differentiate universal effects of dantrolene and AICAR over 24-hours

from state specific effects, the drugs were provided to 2 groups of cells, those receiving EPS and those not receiving EPS. Only two treatments saw significant changes to glucose uptake with EPS vs Without EPS. Cells treated with AICAR saw a significant ($p<0.0001$) decrease in glucose uptake in response to EPS, leading to uptake that was only around 1 quarter of the uptake without EPS at 2.3μM. As expected, control cells saw an increase in glucose uptake with EPS from 3.6 to 8.4μM ($p<0.0001$). Only AICAR treatment also showed a significant difference between EPS and no EPS, with EPS causing a decrease in lactate that corresponded with the decrease in glucose uptake ($p<0.0001$ (**Figure 5.9-1 and 5.9-2**).

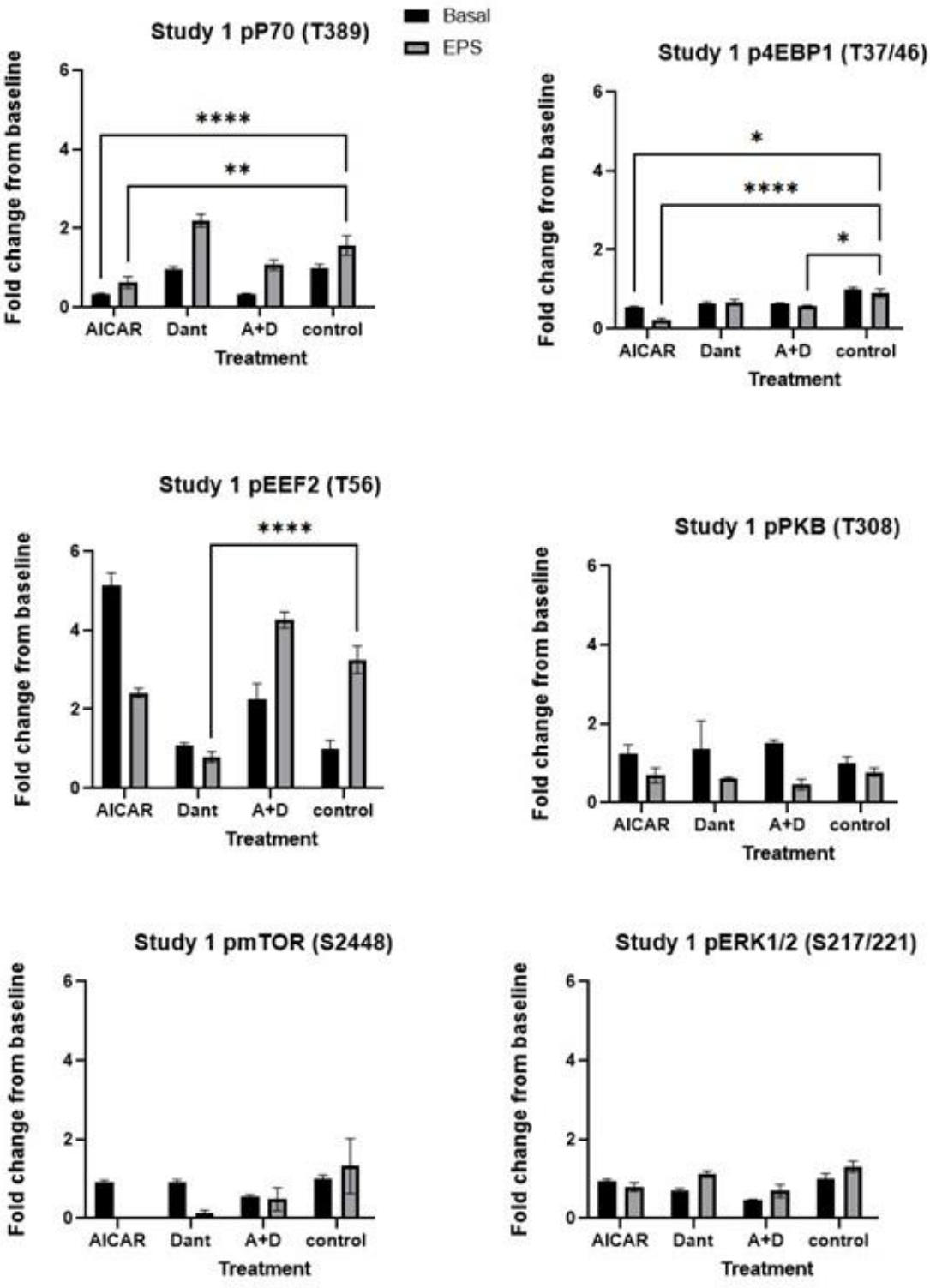


Figure 5.9-1: Changes in anabolic signalling and EEF2, PKB and ERK1/2 phosphorylation and accompanying representative western blots. Cells were either given a rescue drug in the basal state (black bars) or alongside EPS (grey bars).

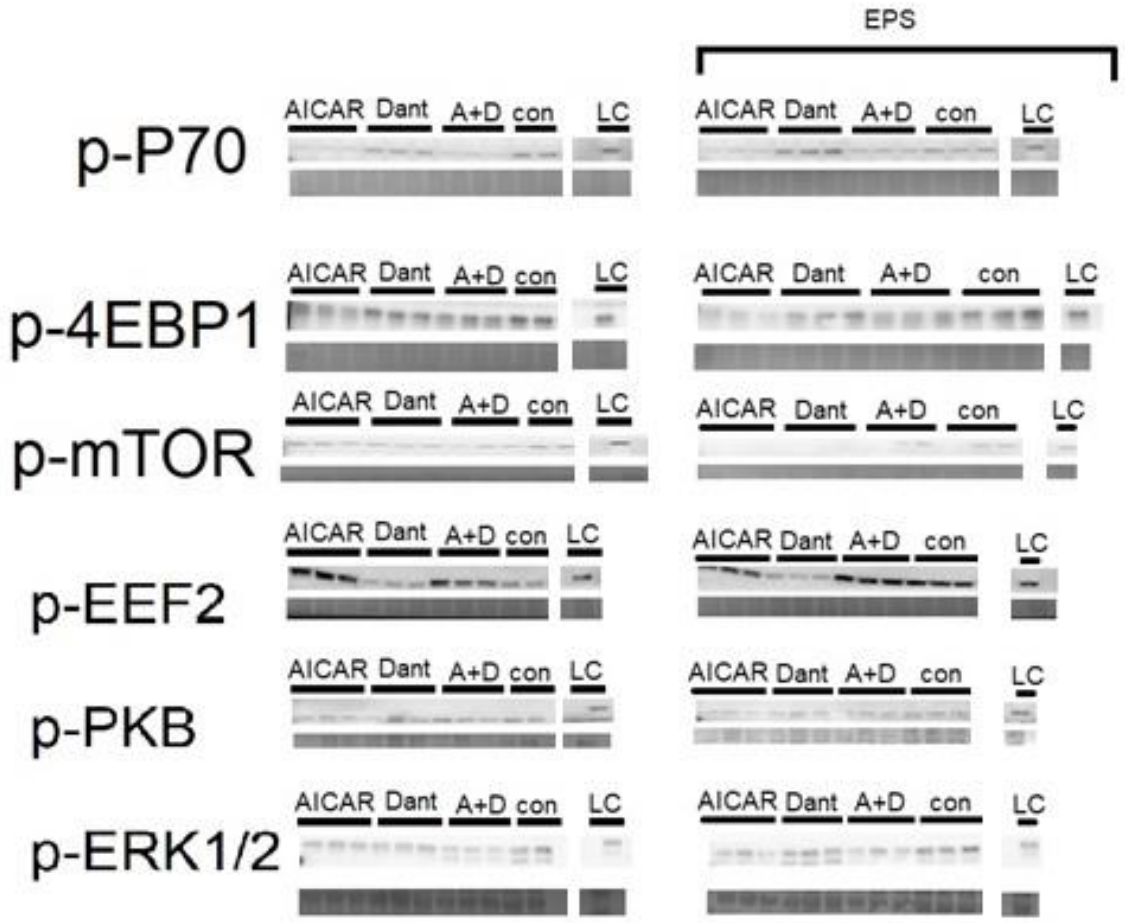


Figure 5.9-2: Representative western blots corresponding to the results shown in figure 5.9-1.

AICAR and AICAR+EPS reduced P70 phosphorylation from control +EPS value ($p<0.001$ and $=0.025$ respectively), whilst dantrolene+ EPS produced a non-significant increase in phosphorylation when compared to controls. As with P70 phosphorylation, both AICAR alone and AICAR+EPS resulted in 4EBP1 phosphorylation that was significantly lower than control + EPS ($p=0.0189$ and <0.0001 respectively). Additionally, AD+ EPS, but not AD alone reduced 4EBP1 phosphorylation ($p=0.0309$). There were no significant differences in mTOR, PKB or ERK1/2 phosphorylation with the addition of any drug + EPS compared to control. Only dantrolene treatment resulted in EEF2 phosphorylation that was significantly less than control whilst EPS was applied ($p<0.0001$). Whilst AD and control saw an increase in EEF2 phosphorylation with EPS application ($p= 0.0003$, 0.0003 and <0.0001 respectively), AICAR alone showed a decrease in phosphorylation with EPS application ($p<0.0001$).

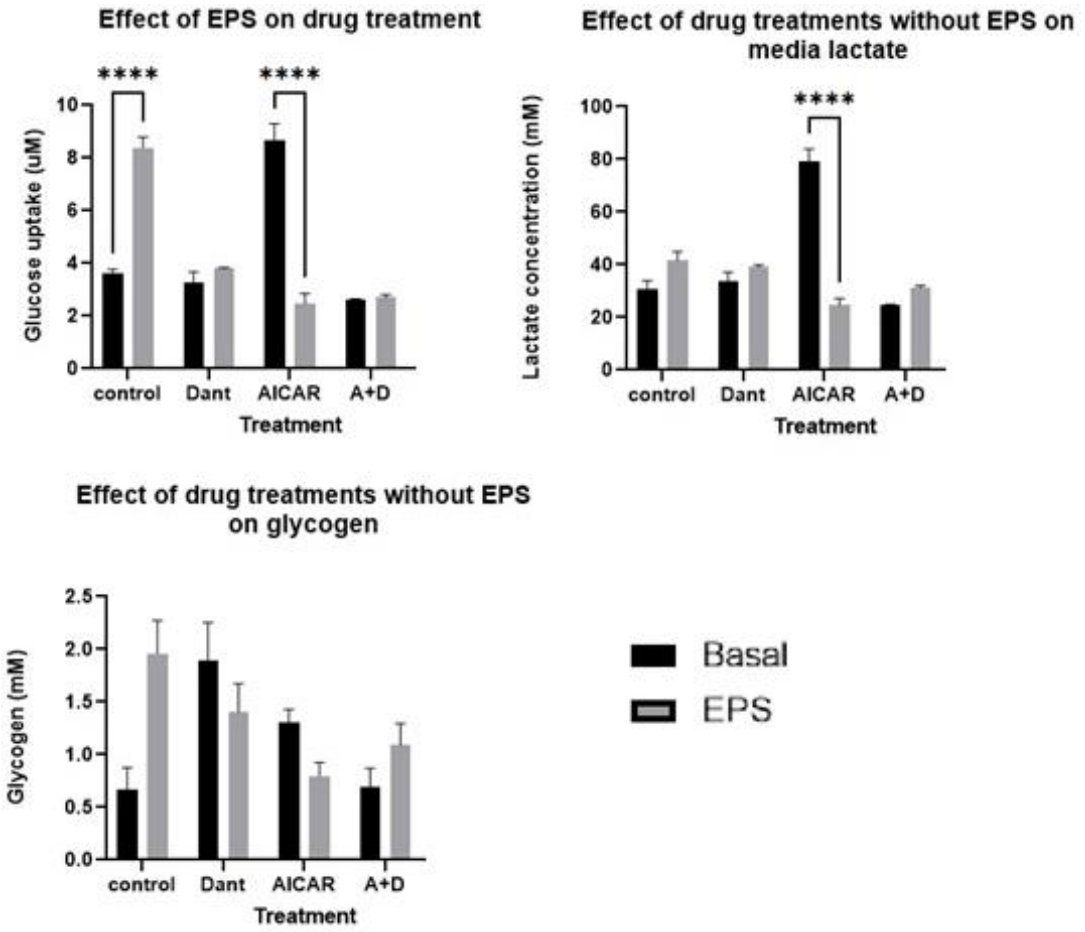


Figure 5.12: Graphs of the changes in glucose uptake, media lactate (lactate output) and Glycogen when cells were treated with a single rescue drug in the presence or absence of EPS for a 24-hour duration. Error bars indicate means with SEM. N=6 (lactate) n= 3 (glycogen and glucose uptake).

5.3 Discussion

After treating the previously established disuse model with drugs targeted to correct the increase in markers of high cytosolic calcium and inactivation of the contraction stimulated glucose uptake pathway (Dantrolene and AICAR) alongside EPS, we found that none of the drugs were able to restore glucose uptake. In fact, the opposite was shown, in which glucose uptake was lowered beyond CB treatment, which itself was lower than baseline uptake. However, full or partial restoration of lactate output was possible. Additionally, we saw that even in the absence of CPA and blebbistatin, EPS completely prevented AICAR from increasing glucose uptake, whilst dantrolene did not significantly change glucose uptake or lactate output, regardless of EPS presence.

5.3.1 Dantrolene treatment

Dantrolene was expected to rectify the changes in calcium handling brought about by CPA which prevents re-uptake by inhibition of the SERCA2 calcium pump. Dantrolene achieves this by preventing passage of calcium through the ryanodine receptor to the cytoplasm. The large reduction in EEF2 phosphorylation at threonine-56 that was not seen with AICAR treatment indicates that calcium leak was prevented as EEF2 phosphorylation is solely controlled via EEF2K - a CamKII sensitive kinase (Hizli *et al*, 2013). Despite the importance of CamKII as a regulator within the EEF2 pathway recent research suggests that CamKII itself has minimal if any *direct* effect on glucose uptake in skeletal muscle due to low abundance or even absence in adult mouse muscle (Negoita *et al*, 2023). However, it remains true that EEF2 activation is impacted by calcium concentration and there is the possibility of other CamK isoforms such as IV having an effect on glucose uptake in skeletal muscle (Shi *et al*, 2017; Ryder *et al*, 2005). Preventing calcium dysregulation negates some of the dysfunction brought about by CB treatment, and this was reflected in glucose uptake during dantrolene+EPS treatment that was higher than AICAR+EPS treatment.

5.3.2 AICAR treatment

That AICAR+EPS treatment of cells treated with CB did not fully restore glucose uptake which is consistent with the lack of contractile ability of CB treated cells as well as the ability of blebbistatin to interfere with stretch derived glucose uptake as well as contraction derived uptake (Saito *et al*, 2016). What is surprising is that the treatment intended to restore glucose uptake actually depressed glucose uptake below the level of what the disuse model achieved. Whilst it is tempting to ascribe this as an effect or interaction of either blebbistatin or CPA and AICAR, the fact that EPS of AICAR treated cells prevented an increase in glucose uptake in the absence of CPA or blebbistatin suggests that the interaction is between EPS and AICAR. Though both exert effects through AMPK, the downstream signalling of EPS and AICAR differs in the exact Rab GTPases that are needed, with EPS requiring recruitment of Rab8a, 13 and 14, whilst AICAR recruits Rab8a and 14 (Li *et al*, 2018). Though not a concrete explanation as to how this may have caused EPS to prevent AICAR induced glucose uptake, it is possible that elements present in this stage of signalling play a role, as there are GTPases such as 8b which have been identified, but have no identified purpose (Benninghoff *et al*, 2020). Additionally, both EPS and AICAR result in endogenous IL-6 vesicle depletion (Hojman *et al*, 2019; Hernández-Albors *et al*, 2019), IL-6 interacts with AMPK as part of the normal sensing of energy and stress state of the cell (Lauritzen *et al*, 2013). It may be possible that the combination of EPS and AICAR caused disturbances in IL-6 outside the range- either of duration or magnitude- of what is normally seen in muscle, resulting in the unexpected lack of stimulation of glucose uptake stemming from changes at the mitochondrial level (Abid *et al*, 2020). However, this argument is difficult to explain with existing literature given that IL-6 secretion is tied to exercise intensity and by extension glycogen store size/ depletion (Steensberg *et al*, 2001). As we did not see any significant changes in glycogen, it is unlikely that IL-6 perturbation caused the depressed

glucose uptake seeing as even relatively extended incubations of IL-6 in muscle resulted in glucose uptake increasing (Kistner *et al*, 2022).

Lastly it is possible that the prolonged EPS protocol elevated cytosolic calcium levels sufficiently to create an environment in which chronic calcium signalling induced effects prevented AMPK phosphorylation, which has been shown before to prevent glucose uptake induced by AMPK manipulation (Park *et al*, 2011).

5.3.4 AICAR+dantrolene treatment

AICAR+EPS and AICAR+dantrolene+EPS showed greater suppression of glucose uptake and lactate output than the other two treatments. It remains unclear where the additional decrease in glucose uptake seen in AICAR and AICAR+ dantrolene comes from. It can be assumed that AICAR is being taken into the cell and activating AMPK as both AICAR+EPS and AICAR+ dantrolene and EPS show reduction in mTOR phosphorylation, which would be a natural outcome stemming from increased AMPK signalling given the antagonism between mTOR and AMPK (Mounier *et al*, 2011). Therefore, the disruption in signalling that would otherwise lead to glucose uptake must lie downstream of AMPK activation.

The fact that AICAR+ dantrolene treatment reduced glucose uptake further than dantrolene alone does not necessarily invalidate the idea that dantrolene reduced cytoplasmic calcium concentration but can be explained by a negative interaction between dantrolene and AICAR. Dantrolene is known to inhibit the effects of AICAR by preventing AMPK phosphorylation as well as prevention of the upregulation of mRNA transcripts for MCT1 and MCT4 (Takimoto *et al*, 2013). Lack of increase in glucose uptake could be caused if the decrease induced by

reduction in AMPK activity was greater than the increase that is derived from preventing chronic calcium leak using dantrolene. Alternatively, the large increase in ERK1/2 phosphorylation seen with dantrolene treatment but not with AD treatment may be related to the difference in glucose uptake. Possible evidence for inhibition of dantrolene's effects by AICAR is that whilst AICAR alone did not have much effect on ERK1/2 phosphorylation and dantrolene produced a large increase, AD heavily suppressed ERK1/2 phosphorylation. In pancreatic β-cells and Chinese hamster ovary, sustained ERk1/2 phosphorylation at serine 217 is a result of GLP-1 agonism (Ehses *et al*, 2002). Overall GLP-1 agonism has beneficial effects that extend to muscle by improving vasculature (Mabilleau *et al,* 2018). A direct effect in c2c12 involving increased production of cAMP has been described with some GLP agonists (Hong *et al*, 2019). Given the involvement of ERK1/2 ser217 phosphorylation in both cases, it is therefore possible that dantrolene is indirectly producing a similar effect.

Throughout the previous chapters, the media lactate concentration has always followed glucose uptake as measured by 2-deoxyglucose-6-phosphate accumulation. Lactate is exported from the cell when the rate of pyruvate formation from any sources outpaces the rate of breakdown of pyruvate in the mitochondria (Stallknecht *et al*, 1998). At the very least, an increase in media lactate is indicative of an increase in glycolysis (Schmiedeknecht, *et al*, 2022). *In vivo* skeletal muscle lactate production is also associated with utilization of glycogen stores, however, we did not record any changes in cell glycogen content that reached significance. An explanation for this might be that the super physiological concentrations of glucose present in high glucose media were sufficient to sustain a favorable AMP:ATP ratio, preventing the activation of glycogen phosphorylase as well as phosphofructokinase which would be responsible for liberation of glucose from glycogen (Hardie, 2011). Based on this understanding of the generation of lactate, it can be assumed that increasing lactate concentration in the

media is reflective of an increase in glucose metabolism, which is why it increases in tandem with glucose uptake. This interpretation suggests that glucose metabolism - with the exception of uptake- was increased by all of the treatments +EPS (AICAR, dantrolene, AICAR+ dantrolene).

5.3.6 EPS does not improve the ability of selected drugs to increase glucose uptake in normal baseline cells.

In order to differentiate effects of EPS from drug induced effects we provided cells with either AICAR, dantrolene or AICAR+ dantrolene. This was repeated with cells that also received EPS alongside the drug treatment. No CPA or blebbistatin was applied. Our finding that EPS in conjunction with drugs did not improve glucose uptake but in fact reduced glucose uptake is surprising, particularly for AICAR, which had been expected to show a greater increase than drugs alone given that EPS should impact the AMP/ADP to ATP ratio. However, an interesting pattern is found in the two highest glucose uptake conditions: AICAR without EPS and EPS with no drug treatment. Whilst AICAR without EPS had both a high glucose uptake and high media lactate concentration, EPS alone had a high glucose uptake but only a moderate increase in media lactate. Furthermore EPS alone had a relatively large glycogen store, measured at 2.0 mM, whilst AICAR without EPS had a glycogen content of 1.3mM. This may be indicative of the fate of glucose taken up by each method of stimulation, oxidation and storage respectively. Presumably, this difference stems from the lack of physical movement present in cells treated only with AICAR, whilst EPS produces both movement and AMPK phosphorylation. Whilst the increase in glycogen content with EPS is not significant, it is a departure from the expected result as glycogen is usually depleted during strenuous or prolonged exercise (Manabe *et al*,

2012). It is possible that sustained phosphorylation of AMPK as a result of contraction resulted in buildup of glucose-6-phosphate which then goes on to activate glycogen synthase directly (Hunter *et al*, 2011).

Paired with the increase in glucose uptake with AICAR treatment was an increase in EEF2, suggesting that cytosolic calcium concentrations increased with AICAR alone, but not with AICAR+ EPS, as EEF2 phosphorylation with AICAR+EPS was less than half that of EPS alone ($P<0.0001$). Interestingly, whilst dantrolene, with and without EPS kept EEF2 phosphorylation low, this was not the case when administered alongside AICAR. This supports the earlier assertion that as well as the known ability of dantrolene to blunt the effects of AICAR, the reverse may also be true. Changes in anabolic signalling when drugs were applied alongside EPS were as expected, with a depression in both 4EBP1 and P70 phosphorylation likely derived from AMPK activation. Indeed, mTOR phosphorylation was reduced below detectable limits by application of AICAR alone and was depressed by AICAR+EPS, dantrolene, dantrolene+ EPS, AD and AD+ EPS.

5.4 Limitations

One of the limitations of the method chosen to replicate disuse was that the binding of blebbistatin is irreversible. Once applied it is not possible for the myotubes to regain the ability to contract. Therefore, it can be considered that any stimulation of glucose uptake that is dependent on contraction- rather than change in AMP:ATP ratio- will not be reproduced by the intervention chosen to rescue the disuse phenotype and so we will not be able to gauge the degree to which the physical movement component of contraction stimulates glucose uptake independent of energy or stress perception.

Another imitation of this experiment is the difficulty in determining where in the time course of acute to chronic the 24-hour timepoint is. Calcium signalling and downstream changes in muscle are by nature rapid as skeletal muscle must respond to changing conditions that can change from inactive and fasting to active fasting and then to fed all within a matter of minutes or hours and there is also evidence that the speed and duration of transients are part of the adaptation of muscle to physical activity (Boulware and Marchant, 2008; Salanova *et al*, 2013; Gejl *et al*, 2020; Tavi and Westerblad, 2011).

One of the reasons for choosing to attempt to restore contraction mediated glucose uptake rather than bolstering insulin dependent pathways is that C2C12 cell's glucose uptake in response to insulin is low when compared to L6 or other muscle cell models (Abdelmoez *et al*, 2020). The drawback to this is that *in vivo* both the contractile and insulin dependent pathways function together to produce a more complete level of glycaemic control than can be recreated in a rodent model currently. Of course, drawback is paired with the advantage of removing inter individual variability as well as the fact that we aimed to determine the effect of exercise specifically on glucose uptake.

5.5 Conclusion

The results above show that whilst it is possible to simulate key aspects of muscle disuse such as depression in glucose uptake, lactate output and anabolic signalling and calcium dysregulation with the application of CPA and blebbistatin it is not possible to restore normal function to C2C12 myotubes with any combination of AICAR, dantrolene and EPS. In fact,

glucose uptake is suppressed further by the addition of these drugs. The results highlight the importance of cytosolic calcium in the maintenance of normal glucose control. They also make apparent potential incompatibility between chronic effects of drugs intended to target speculated intervention points in the signalling chain that propagates the effects of inactivity. Despite the inability to restore glucose uptake, the findings related to the established model offer scope for future work to develop the model in a way that might provide useful information for our understanding of the development of disuse related insulin resistance. The first avenue is direct imaging or measurement of calcium transients at specific timepoints during the application of drugs to the cells, which might elucidate the key subcellular locations that play a role in within the first 24 hours as well as when they occur. Secondly, future work might focus on isolating the contents of vesicles containing relevant myokines in order to understand the changing that occurs in the myokine profile and if they match sufficiently well with what is seen in muscle disuse *in vivo*.

6.0 General discussion

With the recognition of the importance of inactivity as a major factor in the formation of insulin resistance and subsequent health issues such as type 2 diabetes (Booth *et al*, 2012; Lavie *et al*, 2019), there has been an increasing demand for pharmaceutical interventions for the management of blood glucose independently from insulin (Gubert and Hannan, 2021; Narkar *et al*, 2008). In order to research possible pharmaceutical countermeasures against inactivity a readily available model of the condition in skeletal muscle is required. This thesis presents the work carried out to attempt to create, validate and utilize such a model, using the C2C12 cell line.

6.1.1 Validation of the measurement of 2-Deoxyglucose uptake in C2C12 with EPS

Analogs of glucose have seen use as tracers for some time, with detection achieved either by scintillation counter or by quantification of fluorescence (Fatangare and Svatos, 2016). We utilized the non- metabolizable nature of 2-Deoxy-D-glucose in conjunction with triple quadrupole GC-MS to detect phosphorylated 2-DG. As 2-DG is a known inhibitor of glycolysis (Ralser *et al*, 2008) it would be troublesome for the development of any model using this technique, if glucose metabolism were to be disturbed by the tracer used to detect uptake. We showed that at a low dose of 25µM 2-DG there was no inhibition of glycolysis as a result of the tracer's uptake. We also demonstrated that a higher dose of 200µM is not suitable for tracing in C2C12 cells. This is somewhat surprising as previous work had shown that in human primary muscle, relatively high doses of 250µM and 4mM could be provided without causing inhibition of hexokinase or depletion of ATP (Jacobs *et al*, 1990).

Additionally, verification of the contraction of C2C12 cells in response to EPS in our hands was necessary as there are many factors which could influence the ability of cells to contract (unpublished observation) as well as the response that cells would have to contraction. This refers to the contents of the media as well as the growth conditions such as regularity of media changes. For example, we initially found that the presence of amphotericin B, a commonly used anti-fungal agent in tissue culture- prevented the attachment and proliferation of myoblasts. Whilst the pulse train settings we used were not novel, where we had access to points of comparison, they induced appropriate changes in glucose uptake and lactate output, allowing us to gauge that the myotubes were metabolically "normal" for their type.

6.1.2 Effect of varying frequency of intermittent EPS on glucose uptake and related signalling.

As we were unable to identify a difference between intermittent EPS protocols as a result of no change in glucose uptake or lactate output, we can only highlight the importance of maintaining electrical input to myotubes throughout the experimental period. Though there has been much made of the ability of acute bouts of exercise to elicit beneficial effects such as increased glucose uptake for at least 3 hours post exercise (Soo *et al*, 2023) and improvements in measurements of glycemia (Babir *et al*, 2023), but from the measurements we made, we were unable to recreate this effect in C2C12 cells. This may be caused by the effects relying on cross-talk between tissues, or it may be that the post exercise glucose uptake response is not as strong in rodents, though this second explanation is not supported by existing evidence (Ryder *et al*, 1999), with 3 hours of swimming elevating glucose uptake even after 5 hours of resting. We suggested that the explanation for the lack of change in glucose uptake was impairment of the signalling pathway due to disassembly of sarcomeres or other key areas related to contraction, but were unable to verify this with the measurements we had made. This prompts us to ask, how quickly does this disassembly process eradicate changes to glucose uptake if 12 hours of contraction followed by 12 hours of rest showed no difference in uptake from resting cells?

That there were changes in anabolic signalling in response to intermittent EPS confirms that electrical current was indeed reaching the myotubes, however the interpretation of the resulting patterns was difficult. Even accounting for a delay in harvesting it would be expected that at the end of the change, signalling would normalize towards the basal state, however, even patterns of contraction that we had expected to be highly similar did not show matching changes. The 1E23, 2E24 and 3E24 series were expected to produce similar results as they

differed only slightly, however 1 hour contraction produced no change in 4EBP1 phosphorylation, 2 hours contraction produced a significant fall and 3 hours produced a significant increase in 4EBP1. Almost all of the work done on intermittent Electrical pulse stimulation uses human participants who undergo NMES (Neuromuscular Electrical stimulation) with fatigue and pain related measurements rather than biochemical changes (Matsunaga *et al*, 1999; Russ *et al*, 2002). Unfortunately, much of the work that has been done in rodents compares response to high and low frequency EPS against one another rather than to a baseline control or otherwise assesses functional outcomes as in humans (tsutaki *et al*, 2013; Dalise *et al*, 2017; Ichihara *et al*, 2009). This makes drawing parallels to the literature difficult as there are no 1 to 1 comparisons to be had.

6.1.3 Establishing a muscle disuse phenotype in vitro using CPA and blebbistatin

The importance of calcium handling in the function of skeletal muscle has long been appreciated, however the idea that cytosolic calcium could regulate muscle health is relatively new as much of the work before now concerned calcium transients that were sufficiently high to induce contraction and how modifications to calcium handling can influence the contraction rate and fatiguability of muscle (Gordon *et al*, 2000; Taylor *et al*, 1982). Findings that serum calcium levels (Massry and Smogorzewski, 1997) as well as cytosolic calcium (Hyatt and Powers, 2020) can be disturbed during diabetes and bedrest led us to believe that given our understanding of calcium as a regulator of calpains, reproducing a condition of elevated calcium in the absence of contraction may reproduce disuse-like changes within cultured myotubes. Our finding that application of 100µM CPA and 10µM blebbistatin will prevent EPS

induced glucose uptake and lactate output is useful for future skeletal muscle study and confirms findings in rat cardiomyocytes (Ruben *et al*, 2010). More interesting is the finding that anabolic signalling changes that occur in response to this treatment mimic disuse in many ways, with biomarkers CALM1 and ATP2A1 suggesting elevated calcium was achieved. We confirmed suppression of P70 and ERK1/2 phosphorylation, which is in line with current understanding of disuse (Rudrappa *et al*, 2016; Nakanishi *et al*, 2021). With some further development, this model could be useful for the study of different interventions intended to rescue glucose uptake as well as protein synthesis, such as enhanced amino acid provision or novel natural products.

6.1.4 Attempted treatment of the CB model with AICAR and Dantrolene in conjunction with EPS

The inability of dantrolene or AICAR to revert the impairment of glucose uptake brought about by treatment with blebbistatin and CPA demonstrates a flaw with the choice of blebbistatin for preventing contraction and that is the irreversibility of the binding. We attempted to circumvent this and expected that a portion of contraction related glucose uptake could be retained by stimulating the downstream pathway with AICAR, however the results indicate that this is seemingly not the case. Exactly why this is not the case remains unclear, however It may be that the force transduction element of the pathway that responds to contraction (and thus passive stretch) (Kerris *et al*, 2019) was impaired by blebbistatin, as non-muscle myosin can be effected by blebbistatin (Horváth *et al*, 2020).

Alternatively, it may not be a side effect of blebbistatin that prevented restoration of glucose uptake by pharmaceutical intervention. We found that EPS prevented AICAR from exhibiting it's glucose uptake stimulatory effect on C2C12 myotubes that were not treated with any other

drugs. We could not find a satisfactory explanation for this in the literature, so it may be that an as yet unknown signalling conflict between EPS and AICAR prevented glucose uptake restoration, as when attempting rescue of the disuse model EPS was applied under the assumption that the effects of EPS and AICAR would be additive as a result of both shifting the perceived AMP:ATP ratio towards depletion of ATP by changing the phosphorylation of AMPK (Višnjić *et al*, 2021).

6.2. Future considerations and conclusion

6.2.1 Validation of the measurement of 2-Deoxyglucose uptake in C2C12 with EPS

Though we established that 25µM 2DG was suitable for tracing glucose in c2c12 cells in the basal and EPS stimulated state over 24 hours, we did not attempt to find an upper limit to the time that glucose could be measured with this dose. It may be of interest to expand the measurement out to 36 hours, as increasingly the lingering effect of exercise on glycaemic control is being recognized.

6.2.2 Effect of varying frequency of intermittent EPS on glucose uptake and related signalling.

Given the lack of difference between contraction bout frequency and our attributing this to disassembly of sarcomeres and potentially other signal transducing elements, the most immediate modification that could be made to a repeat of this experiment is the addition of a low frequency electrical pulse for the purpose of maintaining sarcomeres. Though it is an

important aspect of contractability, there is little discussion of the use of maintenance pulses even in papers that utilize large gaps between contraction bouts (Manabe *et al*, 2012; Fujita *et al*, 2007), particularly when sarcomere formation is by electrical pulses rather than novel or enhanced substrate (Denes *et al*, 2019). Future work could consider the effect of different pulse lengths and frequency as well as investigating the need or effect of a maintenance pulse as the culture ages as it is known that with additional time in culture C2C12 cells will begin to spontaneously contract, eventually tearing themselves from their substrate (Jensen *et al*, 2020; Denes *et al*, 2019).

6.2.3 Establishing a disuse phenotype in vitro using CPA and blebbistatin

The application of CPA alone was visually confirmed to prevent twitch contraction induced by EPS, as the release of calcium caused by CPA produced a tetanic contraction which was not released. The effects of a lower dose of CPA or initiating calcium leak by other means such as repeated low dose caffeine might show interesting differences which would give insight into the requirement magnitude of calcium leak for disuse-like changes to occur. Following on from this, measurement of changes in calcium sensitive proteases might also be useful in assessment of the model's ability to simulate disuse.

6.2.4 Attempted treatment of the CB model with AICAR and Dantrolene in conjunction with EPS

There are three main questions that arise from the results of the experiments performed in the fifth chapter. Firstly, what are the conditions for the apparent inhibition of AICAR induced glucose uptake by EPS. Second, the permanence of blebbistatin binding to myosin ATPase II limited the degree to which glucose uptake could ever have been recovered. The use of another agent with reversible binding might provide interesting insights based on the

difference in outcome as it could separate effects of electrical current in general from those of contraction. Lastly, the possibility of inhibitory effects between AICAR and dantrolene is of interest, both from the perspective of potential treatments, but also as a research tool. Understanding the interaction between the two would allow for better planning of experiments in the future. An important starting point could be to determine the phosphorylation state of AMPK at Thr^{172} to determine what effect if any AICAR is exerting.

Conclusion

In the course of this work, we demonstrated the suitability of C2C12 myotubes as an exercise responsive platform for the measurement of changes in glucose uptake in our hands. Additionally, we confirmed the viability of a low dose of 2-deoxyglucose for tracing of glucose over a 24-hour period when both EPS and drugs were applied. Though we were able to reproduce disuse-like changes in myotubes including prevention of glucose uptake and reduction in protein synthesis signalling as judged by increased 4EBP1 (T37/46) and reduced P70 (T389) phosphorylation, we were unable to restore glucose uptake pharmaceutically. Though cytosolic calcium was not measured directly CPA treatment was deemed successful in causing persistent calcium elevation due to changes in calcium sensitive mRNA transcripts CALM1 and ATP2A1 as well as phosphorylation of EEF2 (T56). We suggested that the irreversibility of blebbistatin binding was a potential issue with the model and that a reversible substitute could be useful. Despite this we were able to highlight several questions arising from this work, the answers to which may provide useful insights into muscle physiology, particularly, what was the reason for EPS negating AICAR induced glucose uptake? To conclude, the basis of the model appears sound, however the capacity for reverse of the disuse phenotype is important if the model was to be used to determine the efficacy of pharmaceutical treatments for inactivity or immobilization.

References

Abdelmoez, A. M., Sardón Puig, L., Smith, J. A. B., Gabriel, B. M., Savikj, M., Dollet, L., Chibalin, A. V., Krook, A., Zierath, J. R. and Pillon, N. J. (2020) 'Comparative profiling of skeletal muscle models reveals heterogeneity of transcriptome and metabolism', *Am J Physiol Cell Physiol,* 318(3), C615-c626.

Abid, H., Ryan, Z. C., Delmotte, P., Sieck, G. C. and Lanza, I. R. (2020) 'Extramyocellular interleukin-6 influences skeletal muscle mitochondrial physiology through canonical JAK/STAT signalling pathways', *The FASEB Journal,* 34(11), 14458-14472.

Abramowitz, M. K., Hall, C. B., Amodu, A., Sharma, D., Androga, L. and Hawkins, M. (2018) 'Muscle mass, BMI, and mortality among adults in the United States: A population-based cohort study', *PLOS ONE,* 13(4), e0194697.

Abu Bakar, M. H. and Tan, J. S. (2017) 'Improvement of mitochondrial function by celastrol in palmitate-treated C2C12 myotubes via activation of PI3K-Akt signalling pathway', Biomed Pharmacother, 93, 903-912.

Adams, O. P. 'The impact of brief high-intensity exercise on blood glucose levels', (1178-7007 (Print)).

Adeva-Andany, M. M., González-Lucán, M., Donapetry-García, C., Fernández-Fernández, C. and Ameneiros-Rodríguez, E. (2016a) 'Glycogen metabolism in humans', *BBA Clin,* 5, 85-100.

Adeva-Andany, M. M., Pérez-Felpete, N., Fernández-Fernández, C., Donapetry-García, C. and Pazos-García, C. (2016b) 'Liver glucose metabolism in humans', *Biosci Rep,* 36(6).

Aft, R. L., Zhang, F. W. and Gius, D. (2002) 'Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death', *British Journal of Cancer,* 87(7), 805-812.

Agrawal, A., Suryakumar, G. and Rathor, R. (2018) 'Role of defective Ca2+ signalling in skeletal muscle weakness: Pharmacological implications', Journal of Cell Communication and Signalling, 12(4), 645-659.

Agrawal, A., Suryakumar, G. and Rathor, R. (2018) 'Role of defective Ca(2+) signalling in skeletal muscle weakness: Pharmacological implications', J Cell Commun Signal, 12(4), 645-659.

Ahtiainen, J. P., Walker, S., Silvennoinen, M., Kyröläinen, H., Nindl, B. C., Häkkinen, K., Nyman, K., Selänne, H. and Hulmi, J. J. (2015) 'Exercise type and volume alter signalling pathways regulating skeletal muscle glucose uptake and protein synthesis', *European Journal of Applied Physiology,* 115(9), 1835-1845.

Akima, H., Kuno, S., Suzuki, Y., Gunji, A. and Fukunaga, T. (1997) 'Effects of 20 days of bed rest on physiological cross-sectional area of human thigh and leg muscles evaluated by magnetic resonance imaging', *J Gravit Physiol,* 4(1), S15-21.

Aledo, J. C., Lavoie, L., Volchuk, A., Keller, S. R., Klip, A. and Hundal, H. S. (1997) 'Identification and characterization of two distinct intracellular GLUT4 pools in rat skeletal muscle: evidence for an endosomal and an insulin-sensitive GLUT4 compartment', *Biochem J,* 325 (Pt 3)(Pt 3), 727-32.

Alibegovic, A. C., Højbjerre, L., Sonne, M. P., van Hall, G., Stallknecht, B., Dela, F. and Vaag, A. (2009) 'Impact of 9 days of bed rest on hepatic and peripheral insulin action, insulin secretion, and whole-body lipolysis in healthy young male offspring of patients with type 2 diabetes', Diabetes, 58(12), 2749-56.

Alison, R. H. and Mark, R. E. (2015) 'Amount and frequency of exercise affect glycaemic control more than exercise mode or intensity', *British Journal of Sports Medicine,* 49(15), 1012.

Al-Khallaf, H. (2017) 'Isocitrate dehydrogenases in physiology and cancer: biochemical and molecular insight', *Cell Biosci,* 7, 37.

Al-Qusairi, L. and Laporte, J. (2011) 'T-tubule biogenesis and triad formation in skeletal muscle and implication in human diseases', *Skelet Muscle,* 1(1), 26.

Alvim, R. O., Cheuhen, M. R., Machado, S. R., Sousa, A. G. and Santos, P. C. (2015) 'General aspects of muscle glucose uptake', An Acad Bras Cienc, 87(1), 351-68.

American Diabetes Association (ADA) Available online at [https://www.diabetes.org/healthy-living/fitness/weekly-exercise-targets]

Amorim, N. M. L., Kee, A., Coster, A. C. F., Lucas, C., Bould, S., Daniel, S., Weir, J. M., Mellett, N. A., Barbour, J., Meikle, P. J., Cohn, R. J., Turner, N., Hardeman, E. C. and Simar, D. (2020) 'Irradiation impairs mitochondrial function and skeletal muscle oxidative capacity: significance for metabolic complications in cancer survivors', *Metabolism-Clinical and Experimental,* 103, 13.

Andersen, J. L., Gruschy-Knudsen, T., Sandri, C., Larsson, L. and Schiaffino, S. (1999) 'Bed rest increases the amount of mismatched fibers in human skeletal muscle', *J Appl Physiol (1985),* 86(2), 455-60.

Andersen, O. E., Nielsen, O. B. and Overgaard, K. (2018) 'Early effects of eccentric contractions on muscle glucose uptake', *Journal of Applied Physiology,* 126(2), 376-385.

Antonescu, C., Thong, F., Niu, W., Karnieli, E. and Klip, A. (2005) 'To be or not to be: Regulation of the Intrinsic Activity of GLUT4', *Current Medicinal Chemistry - Immunology, Endocrine & Metabolic Agents,* 5, 175-187.

Armstrong, D. D. and Esser, K. A. (2005) 'Wnt/beta-catenin signalling activates growth-control genes during overload-induced skeletal muscle hypertrophy', *Am J Physiol Cell Physiol,* 289(4), C853-9.

Asano, R. Y., Sales, M. M., Browne, R. A., Moraes, J. F., Coelho Júnior, H. J., Moraes, M. R. and Simões, H. G. (2014) 'Acute effects of physical exercise in type 2 diabetes: A review', *World J Diabetes,* 5(5), 659-65.

Ashraf MA, Goyal A. Fludeoxyglucose (18F) [Updated 2023 Aug 28]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK557653/

Åslund, A., Bokhari, M. H., Wetterdal, E., Martin, R., Knölker, H. J. and Bengtsson, T. (2021) 'Myosin 1c: A novel regulator of glucose uptake in brown adipocytes', *Mol Metab,* 53, 101247.

Atherton, P. J. and Rennie, M. J. (2006) 'Protein synthesis a low priority for exercising muscle', *J Physiol,* 573(Pt 2), 288-9.

Atherton, P. J. and Smith, K. (2012) 'Muscle protein synthesis in response to nutrition and exercise', J Physiol, 590(5), 1049-57.

Atherton, P. J., Greenhaff, P. L., Phillips, S. M., Bodine, S. C., Adams, C. M. and Lang, C. H. (2016) 'Control of skeletal muscle atrophy in response to disuse: clinical/preclinical contentions and fallacies of evidence', American Journal of Physiology-Endocrinology and Metabolism, 311(3), E594-E604.

Audzeyenka, I., Rogacka, D., Rachubik, P., Typiak, M., Rychłowski, M., Angielski, S. and Piwkowska, A. (2021) 'The PKGIα-Rac1 pathway is a novel regulator of insulin-dependent glucose uptake in cultured rat podocytes', *J Cell Physiol,* 236(6), 4655-4668.

B. B. Yaspelkis, r., Castle, A. L., Farrar, R. P. and Ivy, J. L. (1997) 'Contraction-induced intracellular signals and their relationship to muscle GLUT-4 concentration', *American Journal of Physiology-Endocrinology and Metabolism,* 272(1), E118-E125.

Babir, F. J., Riddell, M. C., Adamo, L. M., Richards, D. L. and Gibala, M. J. (2023) 'The effect of bodyweight exercise on 24-h glycemic responses determined by continuous glucose monitoring in healthy inactive adults: a randomized crossover study', *Scientific Reports,* 13(1), 20884.

Baehr, L., Tunzi, M. and Bodine, S. (2014) 'Muscle hypertrophy is associated with increases in proteasome activity that is independent of MuRF1 and MAFbx expression', *Frontiers in Physiology,* 5, 69.

Balci-Hayta, B., Bekircan-Kurt, C. E., Aksu, E., Dayangac-Erden, D., Tan, E. and Erdem-Ozdamar, S. (2018) 'Establishment of primary myoblast cell cultures from cryopreserved skeletal muscle biopsies to serve as a tool in related research & development studies', *J Neurol Sci,* 393, 100-104.

Barillas, S. R., Watkins, C. M., Wong, M. A., Dobbs, I. J., Archer, D. C., Munger, C. N., Galpin, A. J., Coburn, J. W. and Brown, L. E. (2017) 'Repeated Plyometric Exercise Attenuates Blood Glucose in Healthy Adults', *Int J Exerc Sci,* 10(7), 1076-1084.

Basirun, C., Ferlazzo, M. L., Howell, N. R., Liu, G. J., Middleton, R. J., Martinac, B., Narayanan, S. A., Poole, K., Gentile, C. and Chou, J. (2021) 'Microgravity × Radiation: A Space Mechanobiology Approach Toward Cardiovascular Function and Disease', *Front Cell Dev Biol,* 9, 750775.

Batool, A., Aashaq, S. and Andrabi, K. I. (2019) 'Eukaryotic initiation factor 4E (eIF4E): A recap of the cap-binding protein', *J Cell Biochem,* 120(9), 14201-14212.

Batty, S. R. and Langlais, P. R. (2021) 'Microtubules in insulin action: what's on the tube?', *Trends in Endocrinology & Metabolism,* 32(10), 776-789.

Bell, R. A. V., Al-Khalaf, M. and Megeney, L. A. (2016) 'The beneficial role of proteolysis in skeletal muscle growth and stress adaptation', *Skeletal Muscle,* 6(1), 16.

Belova, S. P., Zaripova, K., Sharlo, K., Kostrominova, T. Y., Shenkman, B. S. and Nemirovskaya, T. L. (2022) 'Metformin attenuates an increase of calcium-dependent and ubiquitin-proteasome markers in unloaded muscle', *Journal of Applied Physiology,* 133(5), 1149-1163.

Bennett, J. A. and Winters-Stone, K. (2011) 'Motivating older adults to exercise: what works?', *Age and Ageing,* 40(2), 148-149.

Benninghoff, T., Espelage, L., Eickelschulte, S., Zeinert, I., Sinowenka, I., Müller, F., Schöndeling, C., Batchelor, H., Cames, S., Zhou, Z., Kotzka, J., Chadt, A. and Al-Hasani, H. (2020) 'The RabGAPs TBC1D1 and TBC1D4 Control Uptake of

Long-Chain Fatty Acids Into Skeletal Muscle via Fatty Acid Transporter SLC27A4/FATP4', *Diabetes*, 69(11), 2281-2293.

Berg, H. E., Dudley, G. A., Haggmark, T., Ohlsen, H. and Tesch, P. A. (1991) 'Effects of lower limb unloading on skeletal muscle mass and function in humans', *Journal of Applied Physiology*, 70(4), 1882-1885.

Berg, J., Tymoczko, J. and Stryer, L., 2002. *Biochemistry*. 5th ed. New York: W.H.Freeman & Co Ltd, pp.699-727.

Berry, M. V. and Geim, A. K. (1997) 'Of flying frogs and levitrons', *European Journal of Physics*, 18(4), 307-313.

Bhaskar, P. T. and Hay, N. (2007) 'The two TORCs and Akt', *Dev Cell*, 12(4), 487-502.

Biensø, R. S., Ringholm, S., Kiilerich, K., Aachmann-Andersen, N. J., Krogh-Madsen, R., Guerra, B., Plomgaard, P., van Hall, G., Treebak, J. T., Saltin, B., Lundby, C., Calbet, J. A., Pilegaard, H. and Wojtaszewski, J. F. (2012) 'GLUT4 and glycogen synthase are key players in bed rest-induced insulin resistance', *Diabetes*, 61(5), 1090-9.

Biensø, R. S., Ringholm, S., Kiilerich, K., Aachmann-Andersen, N. J., Krogh-Madsen, R., Guerra, B., Plomgaard, P., van Hall, G., Treebak, J. T., Saltin, B., Lundby, C., Calbet, J. A., Pilegaard, H. and Wojtaszewski, J. F. (2012) 'GLUT4 and glycogen synthase are key players in bed rest-induced insulin resistance', *Diabetes*, 61(5), 1090-9.

Biolo, G., Di Girolamo, F. G., Fiotti, N., Mearelli, F. and Sarto, P. (2017) 'Exercise-mediated reactive oxygen species generation in athletes and in patients with chronic disease', *Internal and Emergency Medicine*, 12(6), 741-744.

Biressi, S. and Gopinath, S. D. (2015) 'The quasi-parallel lives of satellite cells and atrophying muscle', *Frontiers in Aging Neuroscience*, 7(140).

Biswas, A., Oh, P. I., Faulkner, G. E., Bajaj, R. R., Silver, M. A., Mitchell, M. S. and Alter, D. A. (2015) 'Sedentary time and its association with risk for disease incidence, mortality, and hospitalization in adults: a systematic review and meta-analysis', *Ann Intern Med*, 162(2), 123-32.

Bkaily, G., Al-Khoury, J., Simon, Y. and Jacques, D. (2017) 'Intracellular Free Calcium Measurement Using Confocal Imaging', Methods Mol Biol, 1527, 177-187.

Blind, E., Janssen, H., Dunder, K. and de Graeff, P. A. (2018) 'The European Medicines Agency's approval of new medicines for type 2 diabetes', *Diabetes Obes Metab*, 20(9), 2059-2063.

Blottner, D., Salanova, M., Püttmann, B., Schiffl, G., Felsenberg, D., Buehring, B. and Rittweger, J. (2006) 'Human skeletal muscle structure and function preserved by

vibration muscle exercise following 55 days of bed rest', European Journal of Applied Physiology, 97(3), 261-271.

Bodine, S. C. (2006) 'mTOR Signalling and the Molecular Adaptation to Resistance Exercise', *Medicine & Science in Sports & Exercise,* 38(11).

Bodine, S. C. (2022) 'The role of mTORC1 in the regulation of skeletal muscle mass', *Fac Rev,* 11, 32.

Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J. and Yancopoulos, G. D. (2001) 'Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo', *Nat Cell Biol,* 3(11), 1014-9.

Booth, F. W., Roberts, C. K. and Laye, M. J. (2012) 'Lack of exercise is a major cause of chronic diseases', *Compr Physiol,* 2(2), 1143-211.

Borges, I. B. P., de Oliveira, D. S., Marie, S. K. N., Lenario, A. M., Oba-Shinjo, S. M. and Shinjo, S. K. (2021) 'Exercise Training Attenuates Ubiquitin-Proteasome Pathway and Increases the Genes Related to Autophagy on the Skeletal Muscle of Patients With Inflammatory Myopathies', *J Clin Rheumatol,* 27(6s), S224-s231.

Borror, A., Zieff, G., Battaglini, C. and Stoner, L. (2018) 'The Effects of Postprandial Exercise on Glucose Control in Individuals with Type 2 Diabetes: A Systematic Review', *Sports Medicine,* 48(6), 1479-1491.

Bose, A., Cherniack, A. D., Langille, S. E., Nicoloro, S. M., Buxton, J. M., Park, J. G., Chawla, A. and Czech, M. P. (2001) 'G(alpha)11 signalling through ARF6 regulates F-actin mobilization and GLUT4 glucose transporter translocation to the plasma membrane', *Mol Cell Biol,* 21(15), 5262-75.

Bosi, P. L., Borges, G. D., Durigan, J. L. Q., Cancelliero, K. M. and Silva, C. A. d. (2008) 'Metformin protects the skeletal muscle glycogen stores against alterations inherent to functional limitation', *Brazilian Archives of Biology and Technology,* 51.

Boulware, M. J. and Marchant, J. S. (2008) 'Timing in cellular Ca2+ signalling', *Curr Biol,* 18(17), R769-r776.

Brandt, N., Gunnarsson, T. P., Bangsbo, J. and Pilegaard, H. (2018) 'Exercise and exercise training-induced increase in autophagy markers in human skeletal muscle', *Physiol Rep,* 6(7), e13651.

Breen, L., Stokes, K. A., Churchward-Venne, T. A., Moore, D. R., Baker, S. K., Smith, K., Atherton, P. J. and Phillips, S. M. (2013) 'Two Weeks of Reduced Activity Decreases Leg Lean Mass and Induces "Anabolic Resistance" of Myofibrillar

Protein Synthesis in Healthy Elderly', *Journal of Clinical Endocrinology & Metabolism,* 98(6), 2604-2612.

Brightwell, C. R., Markofski, M. M., Moro, T., Fry, C. S., Porter, C., Volpi, E. and Rasmussen, B. B. (2019) 'Moderate-intensity aerobic exercise improves skeletal muscle quality in older adults', *Transl Sports Med,* 2(3), 109-119.

Brocca, L., Cannavino, J., Coletto, L., Biolo, G., Sandri, M., Bottinelli, R. and Pellegrino, M. A. (2012) 'The time course of the adaptations of human muscle proteome to bed rest and the underlying mechanisms', *J Physiol,* 590(20), 5211-30.

Brooks, G. A. (2009) 'Cell-cell and intracellular lactate shuttles', *J Physiol,* 587(Pt 23), 5591-600.

Brooks, G. A. (2020) 'Lactate as a fulcrum of metabolism', *Redox Biol,* 35, 101454.

Brooks, N. and Myburgh, K., 2012. Prevention of Skeletal Muscle Wasting: Disuse Atrophy and Sarcopenia. In: J. Cseri, ed., *Skeletal Muscle: from Myogenesis to Clinical Relations*. [online] InTech Open, pp.168-179. Available at: <https://www.intechopen.com/books/skeletal-muscle-from-myogenesis-to-clinical-relations> [Accessed 9 February 2020].

Brown, A. E., Jones, D. E., Walker, M. and Newton, J. L. (2015) 'Abnormalities of AMPK Activation and Glucose Uptake in Cultured Skeletal Muscle Cells from Individuals with Chronic Fatigue Syndrome', *PLOS ONE,* 10(4), e0122982.

Brown, E. C., Franklin, B. A., Regensteiner, J. G. and Stewart, K. J. (2020) 'Effects of single bout resistance exercise on glucose levels, insulin action, and cardiovascular risk in type 2 diabetes: A narrative review', *Journal of Diabetes and its Complications,* 34(8), 107610.

Brozinick, J. T. and Birnbaum, M. J. (1998) 'Insulin, but Not Contraction, Activates Akt/PKB in Isolated Rat Skeletal Muscle', *Journal of Biological Chemistry,* 273(24), 14679-14682.

Brozinick, J. T., Jr., Hawkins, E. D., Strawbridge, A. B. and Elmendorf, J. S. (2004) 'Disruption of Cortical Actin in Skeletal Muscle Demonstrates an Essential Role of the Cytoskeleton in Glucose Transporter 4 Translocation in Insulin-sensitive Tissues *', *Journal of Biological Chemistry,* 279(39), 40699-40706.

Bruton, A. (2002) 'Muscle Plasticity: Response to training and detraining', *Physiotherapy,* 88(7), 398-408.

Burch, N., Arnold, A.-S., Item, F., Summermatter, S., Brochmann Santana Santos, G., Christe, M., Boutellier, U., Toigo, M. and Handschin, C. (2010a) 'Electric Pulse Stimulation of Cultured Murine Muscle Cells Reproduces Gene Expression Changes of Trained Mouse Muscle', *PLOS ONE,* 5(6), e10970.

Burch, N., Arnold, A.-S., Item, F., Summermatter, S., Santos, G., Christe, M., Boutellier, U., Toigo, M. and Handschin, C. (2010b) 'Electric Pulse Stimulation of Cultured Murine Muscle Cells Reproduces Gene Expression Changes of Trained Mouse Muscle', *PloS one,* 5, e10970.

Burke, L. M., van Loon, L. J. C. and Hawley, J. A. (2016) 'Postexercise muscle glycogen resynthesis in humans', *Journal of Applied Physiology,* 122(5), 1055-1067.

Burns, A. M., Nixon, A., Mallinson, J., Cordon, S. M., Stephens, F. B. and Greenhaff, P. L. (2021) 'Immobilisation induces sizeable and sustained reductions in forearm glucose uptake in just 24 h but does not change lipid uptake in healthy men', *The Journal of Physiology,* 599(8), 2197-2210.

Campbell, E. L., Seynnes, O. R., Bottinelli, R., McPhee, J. S., Atherton, P. J., Jones, D. A., Butler-Browne, G. and Narici, M. V. (2013) 'Skeletal muscle adaptations to physical inactivity and subsequent retraining in young men', *Biogerontology,* 14(3), 247-59.

Campbell, T. L. and Quadrilatero, J. (2016) 'Data on skeletal muscle apoptosis, autophagy, and morphology in mice treated with doxorubicin', *Data Brief,* 7, 786-93.

Canadian Space Agency Available online at [https://asc-csa.gc.ca/eng/sciences/vascular.asp]

Cantó, C. and Auwerx, J. (2009) 'PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure', *Curr Opin Lipidol,* 20(2), 98-105.

Carson, B. P. (2017) 'The Potential Role of Contraction-Induced Myokines in the Regulation of Metabolic Function for the Prevention and Treatment of Type 2 Diabetes', *Front Endocrinol (Lausanne),* 8, 97.

Cartee, G. D. (2015) 'Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle', *Diabetologia,* 58(1), 19-30.

Carter, S. and Solomon, T. P. J. (2019) 'In vitro experimental models for examining the skeletal muscle cell biology of exercise: the possibilities, challenges and future developments', *Pflügers Archiv - European Journal of Physiology,* 471(3), 413-429.

Carter, S. and Solomon, T. P. J. (2019) 'In vitro experimental models for examining the skeletal muscle cell biology of exercise: the possibilities, challenges and future developments', *Pflügers Archiv - European Journal of Physiology,* 471(3), 413-429.

Carter, S. and Solomon, T. P. J. (2019) 'In vitro experimental models for examining the skeletal muscle cell biology of exercise: the possibilities, challenges and future developments', *Pflugers Arch,* 471(3), 413-429.

Cetin, H., Liu, W., Cheung, J., Cossins, J., Vanhaesebrouck, A., Maxwell, S., Vincent, A., Beeson, D. and Webster, R. (2019) 'Rapsyn facilitates recovery from desensitization in fetal and adult acetylcholine receptors expressed in a muscle cell line', *Journal of Physiology-London,* 597(14), 3713-3725.

Chadt, A. and Al-Hasani, H. (2020) 'Glucose transporters in adipose tissue, liver, and skeletal muscle in metabolic health and disease', *Pflügers Archiv - European Journal of Physiology,* 472(9), 1273-1298.

Challet, C., Maechler, P., Wollheim, C. B. and Ruegg, U. T. (2001) 'Mitochondrial Calcium Oscillations in C2C12 Myotubes*', *Journal of Biological Chemistry,* 276(6), 3791-3797.

Chambers, M. A., Moylan, J. S., Smith, J. D., Goodyear, L. J. and Reid, M. B. (2009) 'Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase', *J Physiol,* 587(Pt 13), 3363-73.

Chang, S., Song, S., Lee, J., Yoon, J., Park, J., Choi, S., Park, J. K., Choi, K. and Choi, C. (2014) 'Phenotypic modulation of primary vascular smooth muscle cells by short-term culture on micropatterned substrate', *PLoS One,* 9(2), e88089.

Chaturvedi, R., Desai, C., Patel, P., Shah, A. and Dikshit, R. K. (2018) 'An evaluation of the impact of antidiabetic medication on treatment satisfaction and quality of life in patients of diabetes mellitus', *Perspect Clin Res,* 9(1), 15-22.

Chen, H. C., Bandyopadhyay, G., Sajan, M. P., Kanoh, Y., Standaert, M., Farese, R. V., Jr. and Farese, R. V. (2002) 'Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-riboside (AICAR)-stimulated glucose transport', *J Biol Chem,* 277(26), 23554-62.

Chen, L., Tuo, B. and Dong, H. (2016) 'Regulation of Intestinal Glucose Absorption by Ion Channels and Transporters', *Nutrients,* 8(1).

Chen, X., Ji, Y., Liu, R., Zhu, X., Wang, K., Yang, X., Liu, B., Gao, Z., Huang, Y., Shen, Y., Liu, H. and Sun, H. (2023) 'Mitochondrial dysfunction: roles in skeletal muscle atrophy', *Journal of Translational Medicine,* 21(1), 503.

Chen, Y., Huang, L., Qi, X. and Chen, C. (2019) 'Insulin Receptor Trafficking: Consequences for Insulin Sensitivity and Diabetes', *Int J Mol Sci,* 20(20).

Chen, Y., Huang, L., Qi, X. and Chen, C. (2019) 'Insulin Receptor Trafficking: Consequences for Insulin Sensitivity and Diabetes', *International Journal of Molecular Sciences,* 20(20), 5007.

Chen, Y., Zhang, J. and Zhang, X. Y. (2015) '2-NBDG as a marker for detecting glucose uptake in reactive astrocytes exposed to oxygen-glucose deprivation in vitro', *J Mol Neurosci,* 55(1), 126-130.

Chien, H. C., Greenhaff, P. L. and Constantin-Teodosiu, D. (2020) 'PPARδ and FOXO1 Mediate Palmitate-Induced Inhibition of Muscle Pyruvate Dehydrogenase Complex and CHO Oxidation, Events Reversed by Electrical Pulse Stimulation', *Int J Mol Sci,* 21(16).

Chopard, A., Hillock, S. and Jasmin, B. J. (2009) 'Molecular events and signalling pathways involved in skeletal muscle disuse-induced atrophy and the impact of countermeasures', *Journal of Cellular and Molecular Medicine,* 13(9B), 3032-3050.

Chopard, A., Lecunff, M., Danger, R., Lamirault, G., Bihouee, A., Teusan, R., Jasmin, B. J., Marini, J. F. and Leger, J. J. (2009) 'Large-scale mRNA analysis of female skeletal muscles during 60 days of bed rest with and without exercise or dietary protein supplementation as countermeasures', Physiological Genomics, 38(3), 291-302.

Chow, L. S., Albright, R. C., Bigelow, M. L., Toffolo, G., Cobelli, C. and Nair, K. S. (2006) 'Mechanism of insulin's anabolic effect on muscle: measurements of muscle protein synthesis and breakdown using aminoacyl-tRNA and other surrogate measures', *American Journal of Physiology-Endocrinology and Metabolism,* 291(4), E729-E736.

Chowdhury, P., Long, A., Harris, G., Soulsby, M. E. and Dobretsov, M. (2013) 'Animal model of simulated microgravity: a comparative study of hindlimb unloading via tail versus pelvic suspension', *Physiol Rep,* 1(1), e00012.

Clark, B. C. (2009) 'In Vivo Alterations in Skeletal Muscle Form and Function after Disuse Atrophy', Medicine & Science in Sports & Exercise, 41(10).

Cline, G. W., Petersen, K. F., Krssak, M., Shen, J., Hundal, R. S., Trajanoski, Z., Inzucchi, S., Dresner, A., Rothman, D. L. and Shulman, G. I. (1999) 'Impaired Glucose Transport as a Cause of Decreased Insulin-Stimulated Muscle Glycogen Synthesis in Type 2 Diabetes', *New England Journal of Medicine,* 341(4), 240-246.

Clough, G. F. and Egginton, S. (2009) 'Vasomotion and insulin-mediated capillary recruitment--part of the explanation?', *J Physiol,* 587(Pt 14), 3407-8.

Colberg, S. R., Hernandez, M. J. and Shahzad, F. (2013) 'Blood glucose responses to type, intensity, duration, and timing of exercise' in *Diabetes Care*, United States: e177.

Cooper, D. M., Barstow, T. J., Bergner, A. and Lee, W. N. (1989) 'Blood glucose turnover during high- and low-intensity exercise', *Am J Physiol,* 257(3 Pt 1), E405-12.

Coughlan, K. A., Valentine, R. J., Ruderman, N. B. and Saha, A. K. (2014) 'AMPK activation: a therapeutic target for type 2 diabetes?', *Diabetes Metab Syndr Obes,* 7, 241-53.

Coutinho, M., Gerstein, H. C., Wang, Y. and Yusuf, S. (1999) 'The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years', *Diabetes Care,* 22(2), 233-240.

Crofts, C. and Wheldon, M. (2015) 'Hyperinsulinemia: A unifying theory of chronic disease?', *Diabesity,* 1, 34.

Crossland, H., Kazi, A. A., Lang, C. H., Timmons, J. A., Pierre, P., Wilkinson, D. J., Smith, K., Szewczyk, N. J. and Atherton, P. J. (2013) 'Focal adhesion kinase is required for IGF-I-mediated growth of skeletal muscle cells via a TSC2/mTOR/S6K1-associated pathway', *American Journal of Physiology-Endocrinology and Metabolism,* 305(2), E183-E193.

Crossland, H., Skirrow, S., Puthucheary, Z. A., Constantin-Teodosiu, D. and Greenhaff, P. L. (2019) 'The impact of immobilisation and inflammation on the regulation of muscle mass and insulin resistance: different routes to similar end-points', J Physiol, 597(5), 1259-1270.

Cunha, T. F., Moreira, J. B. N., Paixão, N. A., Campos, J. C., Monteiro, A. W. A., Bacurau, A. V. N., Bueno, C. R., Ferreira, J. C. B. and Brum, P. C. (2012) 'Aerobic exercise training upregulates skeletal muscle calpain and ubiquitin-proteasome systems in healthy mice', *Journal of Applied Physiology,* 112(11), 1839-1846.

Cuthbertson, D. J., Babraj, J., Smith, K., Wilkes, E., Fedele, M. J., Esser, K. and Rennie, M. (2006) 'Anabolic signalling and protein synthesis in human skeletal muscle after dynamic shortening or lengthening exercise', American Journal of Physiology-Endocrinology and Metabolism, 290(4), E731-E738.

Dahlén, A. D., Dashi, G., Maslov, I., Attwood, M. M., Jonsson, J., Trukhan, V. and Schiöth, H. B. (2022) 'Trends in Antidiabetic Drug Discovery: FDA Approved Drugs, New Drugs in Clinical Trials and Global Sales', *Frontiers in Pharmacology,* 12.

Dalise, S., Cavalli, L., Ghuman, H., Wahlberg, B., Gerwig, M., Chisari, C., Ambrosio, F. and Modo, M. (2017) 'Biological effects of dosing aerobic exercise and neuromuscular electrical stimulation in rats', *Scientific Reports,* 7(1), 10830.

Davis, S. A. and Davis, B. L. (2012) 'Exercise Equipment Used in Microgravity: Challenges and Opportunities', *Current Sports Medicine Reports,* 11(3), 142-147.

Day, K., Shefer, G., Shearer, A. and Yablonka-Reuveni, Z. (2010) 'The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny', *Dev Biol,* 340(2), 330-43.

de Boer, M. D., Seynnes, O. R., di Prampero, P. E., Pišot, R., Mekjavić, I. B., Biolo, G. and Narici, M. V. (2008) 'Effect of 5 weeks horizontal bed rest on human muscle thickness and architecture of weight bearing and non-weight bearing muscles', *European Journal of Applied Physiology,* 104(2), 401-407.

De Meyts P. The Insulin Receptor and Its Signal Transduction Network. [Updated 2016 Apr 27]. In: Feingold KR, Anawalt B, Boyce A, *et al.*, editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK378978/

de Wendt, C., Espelage, L., Eickelschulte, S., Springer, C., Toska, L., Scheel, A., Bedou, A. D., Benninghoff, T., Cames, S., Stermann, T., Chadt, A. and Al-Hasani, H. (2021) 'Contraction-Mediated Glucose Transport in Skeletal Muscle Is Regulated by a Framework of AMPK, TBC1D1/4, and Rac1', *Diabetes,* 70(12), 2796-2809.

Dean L, McEntyre J. The Genetic Landscape of Diabetes [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2004. Chapter 1, Introduction to Diabetes. 2004 Jul 7. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1671/

Deldicque, L., Atherton, P., Patel, R., Theisen, D., Nielens, H., Rennie, M. J. and Francaux, M. (2008) 'Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise', *European Journal of Applied Physiology,* 104(1), 57-65.

Deldicque, L., Theisen, D. and Francaux, M. (2005) 'Regulation of mTOR by amino acids and resistance exercise in skeletal muscle', *Eur J Appl Physiol,* 94(1-2), 1-10.

Denes, L. T., Riley, L. A., Mijares, J. R., Arboleda, J. D., McKee, K., Esser, K. A. and Wang, E. T. (2019) 'Culturing C2C12 myotubes on micromolded gelatin hydrogels accelerates myotube maturation', *Skeletal Muscle,* 9(1), 17.

DeRuisseau, K. C., Shanely, R. A., Akunuri, N., Hamilton, M. T., Van Gammeren, D., Zergeroglu, A. M., McKenzie, M. and Powers, S. K. (2005) 'Diaphragm unloading via controlled mechanical ventilation alters the gene expression profile', Am J Respir Crit Care Med, 172(10), 1267-75.

Dirks, M. L., Miotto, P. M., Goossens, G. H., Senden, J. M., Petrick, H. L., van Kranenburg, J., van Loon, L. J. C. and Holloway, G. P. (2020) 'Short-term bed rest-induced insulin resistance cannot be explained by increased mitochondrial H2O2 emission', *Journal of Physiology-London,* 598(1), 123-137.

Dirks, M. L., Wall, B. T., van de Valk, B., Holloway, T. M., Holloway, G. P., Chabowski, A., Goossens, G. H. and van Loon, L. J. C. (2016) 'One Week of Bed Rest Leads to Substantial Muscle Atrophy and Induces Whole-Body Insulin Resistance in the Absence of Skeletal Muscle Lipid Accumulation', *Diabetes,* 65(10), 2862.

Dobrowolny, G., Aucello, M., Rizzuto, E., Beccafico, S., Mammucari, C., Bonconpagni, S., Belia, S., Wannenes, F., Nicoletti, C., Del Prete, Z., Rosenthal, N., Molinaro, M., Protasi, F., Fanò, G., Sandri, M. and Musarò, A. (2008) 'Skeletal Muscle Is a Primary Target of SOD1G93A-Mediated Toxicity', *Cell Metabolism,* 8(5), 425-436.

Dos Santos, L., Cyrino, E. S., Antunes, M., Santos, D. A. and Sardinha, L. B. (2017) 'Sarcopenia and physical independence in older adults: the independent and synergic role of muscle mass and muscle function', *J Cachexia Sarcopenia Muscle,* 8(2), 245-250.

Dreyer, H. C., Fujita, S., Cadenas, J. G., Chinkes, D. L., Volpi, E. and Rasmussen, B. B. (2006) 'Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle', *J Physiol,* 576(Pt 2), 613-24.

Dreyer, H. C., Fujita, S., Cadenas, J. G., Chinkes, D. L., Volpi, E. and Rasmussen, B. B. (2006) 'Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle', *J Physiol,* 576(Pt 2), 613-24.

Dreyer, H. C., Fujita, S., Cadenas, J. G., Chinkes, D. L., Volpi, E. and Rasmussen, B. B. (2006) 'Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle', The Journal of Physiology, 576(2), 613-624.

Duan, R. and Gallagher, P. J. (2009) 'Dependence of myoblast fusion on a cortical actin wall and nonmuscle myosin IIA', *Developmental Biology,* 325(2), 374-385.

Duchateau, J. (1995) 'Bed rest induces neural and contractile adaptations in triceps surae', Med Sci Sports Exerc, 27(12), 1581-9.

Dutra, M. T., Alex, S., Silva, A. F., Brown, L. E. and Bottaro, M. (2019) 'Antioxidant Supplementation Impairs Changes in Body Composition Induced by Strength Training in Young Women', Int J Exerc Sci, 12(2), 287-296.

Eaton, S. L., Roche, S. L., Llavero Hurtado, M., Oldknow, K. J., Farquharson, C., Gillingwater, T. H. and Wishart, T. M. (2013) 'Total Protein Analysis as a Reliable Loading Control for Quantitative Fluorescent Western Blotting', *PLOS ONE,* 8(8), e72457.

Egerman, M. A. and Glass, D. J. (2014) 'Signalling pathways controlling skeletal muscle mass', *Crit Rev Biochem Mol Biol,* 49(1), 59-68.

Ehses, J. A., Pelech, S. L., Pederson, R. A. and McIntosh, C. H. S. (2002) 'Glucose-dependent Insulinotropic Polypeptide Activates the Raf-Mek1/2-ERK1/2 Module via a Cyclic AMP/cAMP-dependent Protein Kinase/Rap1-mediated Pathway *', *Journal of Biological Chemistry,* 277(40), 37088-37097.

Elkalaf, M., Anděl, M. and Trnka, J. (2013) 'Low glucose but not galactose enhances oxidative mitochondrial metabolism in C2C12 myoblasts and myotubes', *PLoS One,* 8(8), e70772.

Elkalaf, M., Anděl, M. and Trnka, J. (2013) 'Low glucose but not galactose enhances oxidative mitochondrial metabolism in C2C12 myoblasts and myotubes', *PLoS One,* 8(8), e70772.

English, K. L. and Paddon-Jones, D. (2010) 'Protecting muscle mass and function in older adults during bed rest', *Curr Opin Clin Nutr Metab Care,* 13(1), 34-9.

ESA. (2006) *keeping astronauts fit* [Online] .Available at: http://www.esa.int/Science_Exploration/Human_and_Robotic_Exploration/Business/Keeping_astronauts_fit. Accessed 20/01/2020

Evans, P. L., McMillin, S. L., Weyrauch, L. A. and Witczak, C. A. (2019) 'Regulation of Skeletal Muscle Glucose Transport and Glucose Metabolism by Exercise Training', *Nutrients,* 11(10).

Evans, W. J. (2010) 'Skeletal muscle loss: cachexia, sarcopenia, and inactivity', *The American Journal of Clinical Nutrition,* 91(4), 1123S-1127S.

Evers-van Gogh, I. J. A., Alex, S., Stienstra, R., Brenkman, A. B., Kersten, S. and Kalkhoven, E. (2015) 'Electric Pulse Stimulation of Myotubes as an In Vitro Exercise Model: Cell-Mediated and Non-Cell-Mediated Effects', *Scientific Reports,* 5(1), 10944.

Eyal, S. and Derendorf, H. (2019) 'Medications in Space: In Search of a Pharmacologist's Guide to the Galaxy', *Pharm Res,* 36(10), 148.

Fanzani, A., Conraads, V. M., Penna, F. and Martinet, W. (2012) 'Molecular and cellular mechanisms of skeletal muscle atrophy: an update', *J Cachexia Sarcopenia Muscle,* 3(3), 163-79.

Farmawati, A., Kitajima, Y., Nedachi, T., Sato, M., Kanzaki, M. and Nagatomi, R. (2013) 'Characterization of contraction-induced IL-6 up-regulation using contractile C2C12 myotubes', *Endocr J,* 60(2), 137-47.

Farmawati, A., Kitajima, Y., Nedachi, T., Sato, M., Kanzaki, M. and Nagatomi, R. (2013) 'Characterization of contraction-induced IL-6 up-regulation using contractile C2C12 myotubes', Endocrine Journal, 60(2), 137-147.

Fatangare, A. and Svatoš, A. (2016) 'Applications of 2-deoxy-2-fluoro-D-glucose (FDG) in Plant Imaging: Past, Present, and Future', *Frontiers in Plant Science,* 7.

Fearon, K., Strasser, F., Anker, S. D., Bosaeus, I., Bruera, E., Fainsinger, R. L., Jatoi, A., Loprinzi, C., MacDonald, N., Mantovani, G., Davis, M., Muscaritoli, M., Ottery, F., Radbruch, L., Ravasco, P., Walsh, D., Wilcock, A., Kaasa, S. and Baracos, V. E. (2011) 'Definition and classification of cancer cachexia: an international consensus', *Lancet Oncol,* 12(5), 489-95.

Ferrando, B., Gomez-Cabrera, M. C., Salvador-Pascual, A., Puchades, C., Derbre, F., Gratas-Delamarche, A., Laparre, L., Olaso-Gonzalez, G., Cerda, M., Viosca, E.,

Alabajos, A., Sebastia, V., Alberich-Bayarri, A., Garcia-Castro, F. and Vina, J. (2018) 'Allopurinol partially prevents disuse muscle atrophy in mice and humans', *Scientific Reports,* 8, 12.

Ferraro, E., Giammarioli, A. M., Chiandotto, S., Spoletini, I. and Rosano, G. (2014) 'Exercise-induced skeletal muscle remodeling and metabolic adaptation: redox signalling and role of autophagy', *Antioxid Redox Signal,* 21(1), 154-76.

Filip, S., Mokrý, J., Forostyak, O. and Dayanithi, G. (2019) 'Analysis of Ca(2+) signaling mechanisms - our experience on the intercellular communication in muscle remodeling', *Physiol Res,* 68(2), 325-328.

Fillmore, B., Lankford, D. E., Nightingale, J., Herb, E., Pence, C. and Porter, R. (2010) 'Differences in Glucose Uptake Following High Intensity and Low Intensity Cardiovascular Exercise', *The Journal of Strength & Conditioning Research,* 24.

Fitts, R. H., Riley, D. R. and Widrick, J. J. (2001) 'Functional and structural adaptations of skeletal muscle to microgravity', *Journal of Experimental Biology,* 204(18), 3201.

Flores-Opazo, M., McGee, S. L. and Hargreaves, M. (2020) 'Exercise and GLUT4', Exercise and Sport Sciences Reviews, 48(3).

Flynn-Evans, E. E., Barger, L. K., Kubey, A. A., Sullivan, J. P. and Czeisler, C. A. (2016) 'Circadian misalignment affects sleep and medication use before and during spaceflight', *NPJ Microgravity,* 2, 15019.

Foletta, V. C., White, L. J., Larsen, A. E., Leger, B. and Russell, A. P. (2011) 'The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy', *Pflugers Arch,* 461(3), 325-35.

Fontaine, E. (2018) 'Metformin-Induced Mitochondrial Complex I Inhibition: Facts, Uncertainties, and Consequences', *Front Endocrinol (Lausanne),* 9, 753.

Forsyth, R. J., Bartlett, K. and Eyre, J. (1996) 'Dephosphorylation of 2-deoxyglucose 6-phosphate and 2-deoxyglucose export from cultured astrocytes', *Neurochem Int,* 28(3), 243-50.

Frampton, J., Cobbold, B., Nozdrin, M., Oo, H. T. H., Wilson, H., Murphy, K. G., Frost, G. and Chambers, E. S. (2021) 'The Effect of a Single Bout of Continuous Aerobic Exercise on Glucose, Insulin and Glucagon Concentrations Compared to Resting Conditions in Healthy Adults: A Systematic Review, Meta-Analysis and Meta-Regression', *Sports Medicine,* 51(9), 1949-1966.

Franc, S., Dardari, D., Peschard, C., Riveline, J. P., Biedzinski, M., Boucherie, B., Petit, C., Requeda, E., Mistretta, F., Varroud-Vial, M. and Charpentier, G. (2010) 'Can postprandial blood glucose excursion be predicted in type 2 diabetes?', *Diabetes Care,* 33(9), 1913-8.

Francois, M. E., Baldi, J. C., Manning, P. J., Lucas, S. J. E., Hawley, J. A., Williams, M. J. A. and Cotter, J. D. (2014) ''Exercise snacks' before meals: a novel strategy to improve glycaemic control in individuals with insulin resistance', *Diabetologia,* 57(7), 1437-1445.

Frontera, W. R. and Ochala, J. (2015) 'Skeletal Muscle: A Brief Review of Structure and Function', *Calcified Tissue International,* 96(3), 183-195.

Fu, Z., Gilbert, E. R. and Liu, D. (2013) 'Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes', *Curr Diabetes Rev,* 9(1), 25-53.

Fueger, P. T. (2005) 'Glucose phosphorylation as a barrier to muscle glucose uptake', *Clin Exp Pharmacol Physiol,* 32(4), 314-8.

Fujita, S., Rasmussen, B. B., Cadenas, J. G., Grady, J. J. and Volpi, E. (2006) 'Effect of insulin on human skeletal muscle protein synthesis is modulated by insulin-induced changes in muscle blood flow and amino acid availability', *Am J Physiol Endocrinol Metab,* 291(4), E745-54.

Furman, B. L. (2021) 'Streptozotocin-Induced Diabetic Models in Mice and Rats', Current Protocols, 1(4), e78.

Furukawa, S., Chatani, M., Higashitani, A., Higashibata, A., Kawano, F., Nikawa, T., Numaga-Tomita, T., Ogura, T., Sato, F., Sehara-Fujisawa, A., Shinohara, M., Shimazu, T., Takahashi, S. and Watanabe-Takano, H. (2021) 'Findings from recent studies by the Japan Aerospace Exploration Agency examining musculoskeletal atrophy in space and on Earth', *npj Microgravity,* 7(1), 18.

Gandia, P., Saivin, S. and Houin, G. (2005) 'The influence of weightlessness on pharmacokinetics', *Fundamental & Clinical Pharmacology,* 19(6), 625-636.

Gao, S., Durstine, J. L., Koh, H.-J., Carver, W. E., Frizzell, N. and Carson, J. A. (2017) 'Acute myotube protein synthesis regulation by IL-6-related cytokines', *American Journal of Physiology-Cell Physiology,* 313(5), C487-C500.

Gao, Y., Arfat, Y., Wang, H. and Goswami, N. (2018) 'Muscle Atrophy Induced by Mechanical Unloading: Mechanisms and Potential Countermeasures', Frontiers in Physiology, 9.

Gar, C., Rottenkolber, M., Prehn, C., Adamski, J., Seissler, J. and Lechner, A. (2018) 'Serum and plasma amino acids as markers of prediabetes, insulin resistance, and incident diabetes', *Crit Rev Clin Lab Sci,* 55(1), 21-32.

Gaster, M., Poulsen, P., Handberg, A., Schrøder, H. D. and Beck-Nielsen, H. (2000) 'Direct evidence of fiber type-dependent GLUT-4 expression in human skeletal muscle', *American Journal of Physiology-Endocrinology and Metabolism,* 278(5), E910-E916.

Gejl, K. D., Andersson, E. P., Nielsen, J., Holmberg, H. C. and Ørtenblad, N. (2020) 'Effects of Acute Exercise and Training on the Sarcoplasmic Reticulum Ca(2+) Release and Uptake Rates in Highly Trained Endurance Athletes', *Front Physiol,* 11, 810.

Gejl, K. D., Ortenblad, N., Andersson, E., Plomgaard, P., Holmberg, H. C. and Nielsen, J. (2017) 'Local depletion of glycogen with supramaximal exercise in human skeletal muscle fibres', *Journal of Physiology-London,* 595(9), 2809-2821.

Gertz, K., Uhlemann, R., Foryst-Ludwig, A., Barrientos, R. M., Kappert, K., Thöne-Reineke, C., Djoufack, P., Kirschbaum, C., Fink, K. B., Heinz, A., Kintscher, U., Endres, M. and Kronenberg, G. (2018) 'The cytoskeleton in 'couch potato-ism': Insights from a murine model of impaired actin dynamics', *Experimental Neurology,* 306, 34-44.

Glahn, K. P. E., Bendixen, D., Girard, T., Hopkins, P. M., Johannsen, S., Rüffert, H., Snoeck, M. M. and Urwyler, A. (2020) 'Availability of dantrolene for the management of malignant hyperthermia crises: European Malignant Hyperthermia Group guidelines', *British Journal of Anaesthesia,* 125(2), 133-140.

Gombos, Z., Koltai, E., Torma, F., Bakonyi, P., Kolonics, A., Aczel, D., Ditroi, T., Nagy, P., Kawamura, T. and Radak, Z. (2021) 'Hypertrophy of Rat Skeletal Muscle Is Associated with Increased SIRT1/Akt/mTOR/S6 and Suppressed Sestrin2/SIRT3/FOXO1 Levels', *International Journal of Molecular Sciences,* 22(14), 7588.

Gomez-SanMiguel, A. B., Gomez-Moreira, C., Nieto-Bona, M. P., Fernandez-Galaz, C., Villanua, M. A., Martin, A. I. and Lopez-Calderon, A. (2016) 'Formoterol decreases muscle wasting as well as inflammation in the rat model of rheumatoid arthritis', *American Journal of Physiology-Endocrinology and Metabolism,* 310(11), E925-E937.

Goodyear, L. J. and Kahn, B. B. (1998) 'Exercise, Glucose Transport, and Insulin Sensitivity', *Annual Review of Medicine,* 49(1), 235-261.

Gordon, A. M., Homsher, E. and Regnier, M. (2000) 'Regulation of contraction in striated muscle', *Physiol Rev,* 80(2), 853-924.

Goyal R, Singhal M, Jialal I. Type 2 Diabetes. [Updated 2023 Jun 23]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK513253/

Granacher, U., Gollhofer, A., Hortobagyi, T., Kressig, R. W. and Muehlbauer, T. (2013) 'The importance of trunk muscle strength for balance, functional performance, and fall prevention in seniors: a systematic review', *Sports Med,* 43(7), 627-41.

Gregg, V. A. and Milligan, L. P. (1982) 'In vitro energy costs of Na+, K+ATPase activity and protein synthesis in muscle from calves differing in age and breed', *Br J Nutr,* 48(1), 65-71.

Greiwe, J. S., Hickner, R. C., Hansen, P. A., Racette, S. B., Chen, M. M. and Holloszy, J. O. (1999) 'Effects of endurance exercise training on muscle glycogen accumulation in humans', *Journal of Applied Physiology,* 87(1), 222-226.

Gubert, C. and Hannan, A. J. (2021) 'Exercise mimetics: harnessing the therapeutic effects of physical activity', *Nature Reviews Drug Discovery,* 20(11), 862-879.

Güemes, M., Rahman, S. A. and Hussain, K. (2016) 'What is a normal blood glucose?', *Archives of Disease in Childhood,* 101(6), 569.

Guglielmi, V., Vattemi, G., Gualandi, F., Voermans, N. C., Marini, M., Scotton, C., Pegoraro, E., Oosterhof, A., Kósa, M., Zádor, E., Valente, E. M., De Grandis, D., Neri, M., Codemo, V., Novelli, A., van Kuppevelt, T. H., Dallapiccola, B., van Engelen, B. G., Ferlini, A. and Tomelleri, G. (2013) 'SERCA1 protein expression in muscle of patients with Brody disease and Brody syndrome and in cultured human muscle fibers', Mol Genet Metab, 110(1-2), 162-9.

Guo, X., Greene, K., Akanda, N., Smith, A., Stancescu, M., Lambert, S., Vandenburgh, H. and Hickman, J. (2014) 'In vitro Differentiation of Functional Human Skeletal Myotubes in a Defined System', *Biomater Sci,* 2(1), 131-138.

Gusdon, A. M., Callio, J., Distefano, G., O'Doherty, R. M., Goodpaster, B. H., Coen, P. M. and Chu, C. T. (2017) 'Exercise increases mitochondrial complex I activity and DRP1 expression in the brains of aged mice', *Exp Gerontol,* 90, 1-13.

Hajduch, E., Litherland, G. J. and Hundal, H. S. (2001) 'Protein kinase B (PKB/Akt) – a key regulator of glucose transport?', FEBS Letters, 492(3), 199-203.

Hamada, T., Sasaki, H., Hayashi, T., Moritani, T. and Nakao, K. (2003) 'Enhancement of whole body glucose uptake during and after human skeletal muscle low-frequency electrical stimulation', Journal of Applied Physiology, 94(6), 2107-2112.

Hamarsland, H., Moen, H., Skaar, O. J., Jorang, P. W., Rødahl, H. S. and Rønnestad, B. R. (2022) 'Equal-Volume Strength Training With Different Training Frequencies Induces Similar Muscle Hypertrophy and Strength Improvement in Trained Participants', *Frontiers in Physiology,* 12.

Hamburg, N. M., McMackin, C. J., Huang, A. L., Shenouda, S. M., Widlansky, M. E., Schulz, E., Gokce, N., Ruderman, N. B., Keaney, J. F., Jr. and Vita, J. A. (2007) 'Physical inactivity rapidly induces insulin resistance and microvascular dysfunction in healthy volunteers', Arterioscler Thromb Vasc Biol, 27(12), 2650-6.

Hanefeld, M. (2007) 'Pioglitazone and sulfonylureas: effectively treating type 2 diabetes', *International Journal of Clinical Practice,* 61(s153), 20-27.

Hanke, N., Scheibe, R. J., Manukjan, G., Ewers, D., Umeda, P. K., Chang, K.-C., Kubis, H.-P., Gros, G. and Meissner, J. D. (2011) 'Gene regulation mediating fiber-type transformation in skeletal muscle cells is partly glucose- and ChREBP-dependent', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1813(3), 377-389.

Hansen, P. A., Gulve, E. A. and Holloszy, J. O. (1994) 'Suitability of 2-deoxyglucose for in vitro measurement of glucose transport activity in skeletal muscle', *Journal of Applied Physiology,* 76(2), 979-985.

Harder-Lauridsen, N. M., Krogh-Madsen, R., Holst, J. J., Plomgaard, P., Leick, L., Pedersen, B. K. and Fischer, C. P. (2014) 'Effect of IL-6 on the insulin sensitivity in patients with type 2 diabetes', *American Journal of Physiology-Endocrinology and Metabolism,* 306(7), E769-E778.

Hardie, D. G. (2011) 'AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function', *Genes Dev*, 25(18), 1895-908.

Hargreaves, M. and Cameron-Smith, D. (2002) 'Exercise, diet, and skeletal muscle gene expression', *Medicine & Science in Sports & Exercise,* 34(9).

Hargreaves, M. and Spriet, L. L. (2020) 'Skeletal muscle energy metabolism during exercise', *Nature Metabolism*, 2(9), 817-828.

Hawke, T. J. and Garry, D. J. (2001) 'Myogenic satellite cells: physiology to molecular biology', *Journal of Applied Physiology*, 91(2), 534-551.

Hawkins, A. J. S. (1991) 'PROTEIN-TURNOVER - A FUNCTIONAL APPRAISAL', *Functional Ecology*, 5(2), 222-233.

Haxhi, J., Scotto di Palumbo, A. and Sacchetti, M. (2013) 'Exercising for Metabolic Control: Is Timing Important?', *Annals of Nutrition and Metabolism,* 62(1), 14-25.

He, L. (2020) 'Metformin and Systemic Metabolism', *Trends in Pharmacological Sciences*, 41(11), 868-881.

Heer, M., Baecker, N., Wnendt, S., Fischer, A., Biolo, G. and Frings-Meuthen, P. (2014) 'How Fast Is Recovery of Impaired Glucose Tolerance after 21-Day Bed Rest (NUC Study) in Healthy Adults?', *The Scientific World Journal*, 2014, 803083.

Henriquez-Olguin, C., Knudsen, J. R., Raun, S. H., Li, Z. C., Dalbram, E., Treebak, J. T., Sylow, L., Holmdahl, R., Richter, E. A., Jaimovich, E. and Jensen, T. E. (2019) 'Cytosolic ROS production by NADPH oxidase 2 regulates muscle glucose uptake during exercise', *Nature Communications,* 10, 11.

Hernández-Albors, A., G. Castaño, A., Fernández-Garibay, X., Ortega, M., Balaguer-Trias, J. and Ramón-Azcón, J. (2019) 'Microphysiological sensing platform for

an in-situ detection of tissue-secreted cytokines', *Biosensors and Bioelectronics: X,* 2, 100025.

Herranz, R., Anken, R., Boonstra, J., Braun, M., Christianen, P. C., de Geest, M., Hauslage, J., Hilbig, R., Hill, R. J., Lebert, M., Medina, F. J., Vagt, N., Ullrich, O., van Loon, J. J. and Hemmersbach, R. (2013) 'Ground-based facilities for simulation of microgravity: organism-specific recommendations for their use, and recommended terminology', *Astrobiology,* 13(1), 1-17.

Hinkle, J. S., Rivera, C. N. and Vaughan, R. A. (2022) 'AICAR stimulates mitochondrial biogenesis and BCAA catabolic enzyme expression in C2C12 myotubes', *Biochimie,* 195, 77-85.

Hinks, A., Hawke, T. J., Franchi, M. V. and Power, G. A. (2023) 'The importance of serial sarcomere addition for muscle function and the impact of aging', *J Appl Physiol (1985).*

Hirabara, S. M., Curi, R. and Maechler, P. (2010) 'Saturated fatty acid-induced insulin resistance is associated with mitochondrial dysfunction in skeletal muscle cells', *J Cell Physiol,* 222(1), 187-94.

Hizli, A. A., Chi, Y., Swanger, J., Carter, J. H., Liao, Y., Welcker, M., Ryazanov, A. G. and Clurman, B. E. (2013) 'Phosphorylation of eukaryotic elongation factor 2 (eEF2) by cyclin A-cyclin-dependent kinase 2 regulates its inhibition by eEF2 kinase', *Mol Cell Biol,* 33(3), 596-604.

Hizli, A. A., Chi, Y., Swanger, J., Carter, J. H., Liao, Y., Welcker, M., Ryazanov, A. G. and Clurman, B. E. (2013) 'Phosphorylation of eukaryotic elongation factor 2 (eEF2) by cyclin A-cyclin-dependent kinase 2 regulates its inhibition by eEF2 kinase', Mol Cell Biol, 33(3), 596-604.

Hodson, N., West, D. W. D., Philp, A., Burd, N. A. and Moore, D. R. (2019) 'Molecular regulation of human skeletal muscle protein synthesis in response to exercise and nutrients: a compass for overcoming age-related anabolic resistance', *Am J Physiol Cell Physiol,* 317(6), C1061-c1078.

Hoecht, E. M. (2021) 'The missing ‘link’? β-Catenin's role in skeletal muscle glucose uptake during exercise and contraction', *The Journal of Physiology,* 599(21), 4737-4739.

Hojman, P., Brolin, C., Nørgaard-Christensen, N., Dethlefsen, C., Lauenborg, B., Olsen, C. K., Åbom, M. M., Krag, T., Gehl, J. and Pedersen, B. K. (2019) 'IL-6 release from muscles during exercise is stimulated by lactate-dependent protease activity', *American Journal of Physiology-Endocrinology and Metabolism,* 316(5), E940-E947.

Hollingworth, S., Chandler, W. K. and Baylor, S. M. (2006) 'Effects of tetracaine on voltage-activated calcium sparks in frog intact skeletal muscle fibers', *Journal of General Physiology,* 127(3), 291-307.

Holm, L. and Kjaer, M. (2010) 'Measuring protein breakdown rate in individual proteins in vivo', Curr Opin Clin Nutr Metab Care, 13(5), 526-31.

Hong, Y., Lee, J. H., Jeong, K. W., Choi, C. S. and Jun, H. S. (2019) 'Amelioration of muscle wasting by glucagon-like peptide-1 receptor agonist in muscle atrophy', *J Cachexia Sarcopenia Muscle,* 10(4), 903-918.

Hoppeler, H. and Fluck, M. (2002) 'Normal mammalian skeletal muscle and its phenotypic plasticity', *J Exp Biol,* 205(Pt 15), 2143-52.

Horváth, Á. I., Gyimesi, M., Várkuti, B. H., Képiró, M., Szegvári, G., Lőrincz, I., Hegyi, G., Kovács, M. and Málnási-Csizmadia, A. (2020) 'Effect of allosteric inhibition of non-muscle myosin 2 on its intracellular diffusion', *Scientific Reports,* 10(1), 13341.

Hu, F., Li, N., Li, Z., Zhang, C., Yue, Y., Liu, Q., Chen, L., Bilan, P. J. and Niu, W. (2018) 'Electrical pulse stimulation induces GLUT4 translocation in a Rac-Akt-dependent manner in C2C12 myotubes', *FEBS Lett,* 592(4), 644-654.

Huard, C., Martinez, R. V., Ross, C., Johnson, J. W., Zhong, W., Hill, A. A., Kim, R., Paulsen, J. E. and Shih, H. H. (2007) 'Transcriptional profiling of C2C12 myotubes in response to SHIP2 depletion and insulin stimulation', *Genomics,* 89(2), 270-279.

Huchet, C. and Léoty, C. (1993) 'Effects of cyclopiazonic acid on Ca(2+)-activated tension production in skinned skeletal muscle fibres of the ferret', Eur J Pharmacol, 241(1), 41-6.

Hunter, R. W., Treebak, J. T., Wojtaszewski, J. F. and Sakamoto, K. (2011) 'Molecular mechanism by which AMP-activated protein kinase activation promotes glycogen accumulation in muscle', *Diabetes,* 60(3), 766-74.

Hyatt, H. W. and Powers, S. K. (2020) 'Disturbances in Calcium Homeostasis Promotes Skeletal Muscle Atrophy: Lessons From Ventilator-Induced Diaphragm Wasting', *Front Physiol,* 11, 615351.

Hyatt, H. W. and Powers, S. K. (2020) 'The role of calpains in skeletal muscle remodeling with exercise and inactivity-induced atrophy', Int. J. Sports Med., 41(14), 994-1008.

Hyatt, H. W. and Powers, S. K. (2020a) 'Disturbances in Calcium Homeostasis Promotes Skeletal Muscle Atrophy: Lessons From Ventilator-Induced Diaphragm Wasting', Front Physiol, 11, 615351.

Hyatt, H. W. and Powers, S. K. (2020b) 'Disturbances in Calcium Homeostasis Promotes Skeletal Muscle Atrophy: Lessons From Ventilator-Induced Diaphragm Wasting', Front Physiol, 11, 615351.

Hyatt, H., Deminice, R., Yoshihara, T. and Powers, S. K. (2019) 'Mitochondrial dysfunction induces muscle atrophy during prolonged inactivity: A review of the causes and effects', *Arch Biochem Biophys,* 662, 49-60.

Ichihara, K., Venkatasubramanian, G., Abbas, J. J. and Jung, R. (2009) 'Neuromuscular electrical stimulation of the hindlimb muscles for movement therapy in a rodent model', *J Neurosci Methods,* 176(2), 213-24.

Impey, S. G., Hammond, K. M., Shepherd, S. O., Sharples, A. P., Stewart, C., Limb, M., Smith, K., Philp, A., Jeromson, S., Hamilton, D. L., Close, G. L. and Morton, J. P. (2016) 'Fuel for the work required: a practical approach to amalgamating train-low paradigms for endurance athletes', *Physiol Rep,* 4(10).

Inoki, K., Zhu, T. and Guan, K. L. (2003a) 'TSC2 mediates cellular energy response to control cell growth and survival', *Cell,* 115(5), 577-90.

Inoki, K., Zhu, T. and Guan, K. L. (2003b) 'TSC2 mediates cellular energy response to control cell growth and survival', *Cell,* 115(5), 577-90.

Israël, A. (2010) 'The IKK complex, a central regulator of NF-kappaB activation', *Cold Spring Harb Perspect Biol,* 2(3), a000158.

Itagaki, K., Menconi, M., Antoniu, B., Zhang, Q., Gonnella, P., Soybel, D., Hauser, C. and Hasselgren, P. O. (2010) 'Dexamethasone stimulates store-operated calcium entry and protein degradation in cultured L6 myotubes through a phospholipase A(2)-dependent mechanism', Am J Physiol Cell Physiol, 298(5), C1127-39.

Ito, T., Tagawa, Y., Shiba, N., Tanaka, S., Umezu, Y., Yamamoto, T. and Basford, J. R. (2004) 'Development of practical and effective hybrid exercise for use in weightless environment', *Conf Proc IEEE Eng Med Biol Soc,* 6, 4252-5.

Ivy, J. L., Withers, R. T., Van Handel, P. J., Elger, D. H. and Costill, D. L. (1980) 'Muscle respiratory capacity and fiber type as determinants of the lactate threshold', *Journal of Applied Physiology,* 48(3), 523-527.

Iwata, M., Suzuki, S., Hayakawa, K., Inoue, T. and Naruse, K. (2009) 'Uniaxial Cyclic Stretch Increases Glucose Uptake into C2C12 Myotubes through a Signalling Pathway Independent of Insulin-like Growth Factor I', *Horm Metab Res,* 41(01), 16-22.

Jacobs, A. E. M., Oosterhof, A. and Veerkamp, J. H. (1990) '2-Deoxy-d-glucose uptake in cultured human muscle cells', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1051(3), 230-236.

Jensen, J., Rustad Pi Fau - Kolnes, A. J., Kolnes Aj Fau - Lai, Y.-C. and Lai, Y. C. 'The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise', (1664-042X (Electronic)).

Jensen, J., Rustad, P. I., Kolnes, A. J. and Lai, Y. C. (2011) 'The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise', *Front Physiol,* 2, 112.

Jensen, T. E., Angin, Y., Sylow, L. and Richter, E. A. (2014) 'Is contraction-stimulated glucose transport feedforward regulated by Ca2+?', *Exp Physiol,* 99(12), 1562-8.

Jensen, T. E., Sylow, L., Rose, A. J., Madsen, A. B., Angin, Y., Maarbjerg, S. J. and Richter, E. A. (2014) 'Contraction-stimulated glucose transport in muscle is controlled by AMPK and mechanical stress but not sarcoplasmatic reticulum Ca2+ release', *Molecular Metabolism,* 3(7), 742-753.

Jensen, T. E., Sylow, L., Rose, A. J., Madsen, A. B., Angin, Y., Maarbjerg, S. J. and Richter, E. A. (2014) 'Contraction-stimulated glucose transport in muscle is controlled by AMPK and mechanical stress but not sarcoplasmatic reticulum Ca2+ release', Molecular Metabolism, 3(7), 742-753.

Jin, B., Shi, H., Zhu, J., Wu, B. and Geshang, Q. (2020) 'Up-regulating autophagy by targeting the mTOR-4EBP1 pathway: a possible mechanism for improving cardiac function in mice with experimental dilated cardiomyopathy', *BMC Cardiovascular Disorders,* 20(1), 56.

Jones, D. A. (1996) 'High- and low-frequency fatigue revisited', *Acta Physiologica Scandinavica,* 156(3), 265-270.

Jones, N. C., Fedorov, Y. V., Rosenthal, R. S. and Olwin, B. B. (2001) 'ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion', Journal of Cellular Physiology, 186(1), 104-115.

Jones, T. W., Walshe, I. H., Hamilton, D. L., Howatson, G., Russell, M., Price, O. J., Gibson, A. S. and French, D. N. (2016) 'Signalling Responses After Varying Sequencing of Strength and Endurance Training in a Fed State', *Int J Sports Physiol Perform,* 11(7), 868-875.

Jost, P. D. (2008) 'Simulating human space physiology with bed rest', *Hippokratia,* 12 Suppl 1, 37-40.

Joyner, M. J. and Coyle, E. F. (2008) 'Endurance exercise performance: the physiology of champions', *J Physiol,* 586(1), 35-44.

Juel, C. and Halestrap, A. P. (1999) 'Lactate transport in skeletal muscle - role and regulation of the monocarboxylate transporter', *J Physiol,* 517 (Pt 3)(Pt 3), 633-42.

Kalender, A., Selvaraj, A., Kim, S. Y., Gulati, P., Brûlé, S., Viollet, B., Kemp, B. E., Bardeesy, N., Dennis, P., Schlager, J. J., Marette, A., Kozma, S. C. and Thomas, G. (2010) 'Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner', *Cell Metab,* 11(5), 390-401.

Kast, J., Yu, Y., Seubert, C. N., Wotring, V. E. and Derendorf, H. (2017) 'Drugs in space: Pharmacokinetics and pharmacodynamics in astronauts', *European Journal of Pharmaceutical Sciences,* 109, S2-S8.

Katz, J. and Wood, H. G. (1960) 'The use of glucose-C14 for the evaluation of the pathways of glucose metabolism', *J Biol Chem,* 235, 2165-77.

Kawamoto, E., Tamakoshi, K., Ra, S.-G., Masuda, H. and Kawanaka, K. (2018) 'Immobilization rapidly induces thioredoxin-interacting protein gene expression together with insulin resistance in rat skeletal muscle', *Journal of Applied Physiology,* 125(2), 596-604.

Kazyken, D., Magnuson, B., Bodur, C., Acosta-Jaquez, H. A., Zhang, D., Tong, X., Barnes, T. M., Steinl, G. K., Patterson, N. E., Altheim, C. H., Sharma, N., Inoki, K., Cartee, G. D., Bridges, D., Yin, L., Riddle, S. M. and Fingar, D. C. (2019) 'AMPK directly activates mTORC2 to promote cell survival during acute energetic stress', *Sci Signal,* 12(585).

Kelley, D. E., He, J., Menshikova, E. V. and Ritov, V. B. (2002) 'Dysfunction of Mitochondria in Human Skeletal Muscle in Type 2 Diabetes', *Diabetes,* 51(10), 2944.

Kennedy, A., Nirantharakumar, K., Chimen, M., Pang, T. T., Hemming, K., Andrews, R. C. and Narendran, P. (2013) 'Does exercise improve glycaemic control in type 1 diabetes? A systematic review and meta-analysis', *PLoS One,* 8(3), e58861.

Kerris, J. P., Betik, A. C., Li, J. and McConell, G. K. (2018) 'Passive stretch regulates skeletal muscle glucose uptake independent of nitric oxide synthase', *Journal of Applied Physiology,* 126(1), 239-245.

Keshel, T. E. and Coker, R. H. (2015b) 'Exercise Training and Insulin Resistance: A Current Review', J Obes Weight Loss Ther, 5(Suppl 5).

Khan, M. A. B., Hashim, M. J., King, J. K., Govender, R. D., Mustafa, H. and Al Kaabi, J. (2020a) 'Epidemiology of Type 2 Diabetes - Global Burden of Disease and Forecasted Trends', *J Epidemiol Glob Health,* 10(1), 107-111.

Khan, M. A. B., Hashim, M. J., King, J. K., Govender, R. D., Mustafa, H. and Al Kaabi, J. (2020b) 'Epidemiology of Type 2 Diabetes - Global Burden of Disease and Forecasted Trends', *J Epidemiol Glob Health,* 10(1), 107-111.

Khodabukus, A. and Baar, K. (2015) 'Glucose concentration and streptomycin alter in vitro muscle function and metabolism', *J Cell Physiol,* 230(6), 1226-34.

Kim, I.-Y., Suh, S.-H., Lee, I.-K. and Wolfe, R. R. (2016) 'Applications of stable, nonradioactive isotope tracers in in vivo human metabolic research', *Experimental & Molecular Medicine,* 48(1), e203-e203.

Kim, J., Yang, G., Kim, Y., Kim, J. and Ha, J. (2016) 'AMPK activators: mechanisms of action and physiological activities', *Experimental & Molecular Medicine,* 48(4), e224-e224.

Kim, Y., Triolo, M. and Hood, D. A. (2017) 'Impact of Aging and Exercise on Mitochondrial Quality Control in Skeletal Muscle', *Oxid Med Cell Longev,* 2017, 3165396.

Kistner, T. M., Pedersen, B. K. and Lieberman, D. E. (2022) 'Interleukin 6 as an energy allocator in muscle tissue', *Nature Metabolism,* 4(2), 170-179.

Kjøbsted, R., Chadt, A., Jørgensen, N. O., Kido, K., Larsen, J. K., de Wendt, C., Al-Hasani, H. and Wojtaszewski, J. F. P. (2019) 'TBC1D4 Is Necessary for Enhancing Muscle Insulin Sensitivity in Response to AICAR and Contraction', Diabetes, 68(9), 1756-1766.

Knuiman, P., Hopman, M. T. and Mensink, M. (2015) 'Glycogen availability and skeletal muscle adaptations with endurance and resistance exercise', *Nutr Metab (Lond),* 12, 59.

Knuiman, P., Hopman, M. T. E. and Mensink, M. (2015) 'Glycogen availability and skeletal muscle adaptations with endurance and resistance exercise', Nutrition & Metabolism, 12(1), 59.

Kobayashi, S., Bannister, M. L., Gangopadhyay, J. P., Hamada, T., Parness, J. and Ikemoto, N. (2005) 'Dantrolene Stabilizes Domain Interactions within the Ryanodine Receptor*', *Journal of Biological Chemistry,* 280(8), 6580-6587.

Koh, H.-C. E., van Vliet, S., Meyer, G. A., Laforest, R., Gropler, R. J., Klein, S. and Mittendorfer, B. (2021) 'Heterogeneity in insulin-stimulated glucose uptake among different muscle groups in healthy lean people and people with obesity', *Diabetologia,* 64(5), 1158-1168.

Kohn, F. P. M. and Hauslage, J. (2019) 'The gravity dependence of pharmacodynamics: the integration of lidocaine into membranes in microgravity', *NPJ Microgravity,* 5, 5.

Kolega, J. (2004) 'Phototoxicity and photoinactivation of blebbistatin in UV and visible light', Biochemical and Biophysical Research Communications, 320(3), 1020-1025.

Konopka, A. R., Laurin, J. L., Schoenberg, H. M., Reid, J. J., Castor, W. M., Wolff, C. A., Musci, R. V., Safairad, O. D., Linden, M. A., Biela, L. M., Bailey, S. M.,

Hamilton, K. L. and Miller, B. F. (2019) 'Metformin inhibits mitochondrial adaptations to aerobic exercise training in older adults', *Aging Cell,* 18(1), e12880.

Kotani, T., Takegaki, J., Tamura, Y., Kouzaki, K., Nakazato, K. and Ishii, N. (2021) 'The effect of repeated bouts of electrical stimulatio induced muscle contractions on proteolytic signalling in rat skeletal muscle', *Physiological Reports,* 9.

Kovács, M., Tóth, J., Hetényi, C., Málnási-Csizmadia, A. and Sellers, J. R. (2004) 'Mechanism of blebbistatin inhibition of myosin II', J Biol Chem, 279(34), 35557-63.

Krainski, F., Hastings, J., Heinicke, K., Romain, N., Pacini, E., Snell, P., Wyrick, P., Palmer, M., Haller, R. and Levine, B. (2014)' The effect of rowing ergometry and resistive exercise on skeletal muscle structure and function during bed rest', *Journal of Applied Physiology,* 116(12), 1569-1581

Kramer, H. F. and Goodyear, L. J. (2007) 'Exercise, MAPK, and NF-κB signalling in skeletal muscle', *Journal of Applied Physiology,* 103(1), 388-395.

Krause, T., Gerbershagen, M. U., Fiege, M., Weißhorn, R. and Wappler, F. (2004) 'Dantrolene – A review of its pharmacology, therapeutic use and new developments', *Anaesthesia,* 59(4), 364-373.

Kristensen, J. M., Larsen, S., Helge, J. W., Dela, F. and Wojtaszewski, J. F. P. (2013) 'Two Weeks of Metformin Treatment Enhances Mitochondrial Respiration in Skeletal Muscle of AMPK Kinase Dead but Not Wild Type Mice', *PLOS ONE,* 8(1), e53533.

Kristiansen, O. P. and Mandrup-Poulsen, T. (2005) 'Interleukin-6 and Diabetes', *Diabetes,* 54(suppl 2), S114.

Kulkarni, A. S., Peck, B. D., Walton, R. G., Kern, P. A., Mar, J. C., Windham, S. T., Bamman, M. M., Barzilai, N. and Peterson, C. A. (2020) 'Metformin alters skeletal muscle transcriptome adaptations to resistance training in older adults', *Aging,* 12(20), 19852-19866.

Kumar, A., Davuluri, G., Welch, N., Kim, A., Gangadhariah, M., Allawy, A., Priyadarshini, A., McMullen, M. R., Sandlers, Y., Willard, B., Hoppel, C. L., Nagy, L. E. and Dasarathy, S. (2019) 'Oxidative stress mediates ethanol-induced skeletal muscle mitochondrial dysfunction and dysregulated protein synthesis and autophagy', *Free Radical Biology and Medicine,* 145, 284-299.

Kumar, V., Atherton, P., Smith, K. and Rennie, M. J. (2009) 'Human muscle protein synthesis and breakdown during and after exercise', *Journal of Applied Physiology,* 106(6), 2026-2039.

Kumar, V., Atherton, P., Smith, K. and Rennie, M. J. (2009) 'Human muscle protein synthesis and breakdown during and after exercise', *Journal of Applied Physiology,* 106(6), 2026-2039.

Kwon, O. S., Nelson, D. S., Barrows, K. M., O'Connell, R. M. and Drummond, M. J. (2016) 'Intramyocellular ceramides and skeletal muscle mitochondrial respiration are partially regulated by Toll-like receptor 4 during hindlimb unloading', *American journal of physiology. Regulatory, integrative and comparative physiology,* 311(5), R879-R887.

Kwon, O. S., Tanner, R. E., Barrows, K. M., Runtsch, M., Symons, J. D., Jalili, T., Bikman, B. T., McClain, D. A., O'Connell, R. M. and Drummond, M. J. (2015) 'MyD88 regulates physical inactivity-induced skeletal muscle inflammation, ceramide biosynthesis signalling, and glucose intolerance', *Am J Physiol Endocrinol Metab,* 309(1), E11-21.

Langone, F., Cannata, S., Fuoco, C., Lettieri Barbato, D., Testa, S., Nardozza, A. P., Ciriolo, M. R., Castagnoli, L., Gargioli, C. and Cesareni, G. (2014) 'Metformin Protects Skeletal Muscle from Cardiotoxin Induced Degeneration', *PLOS ONE,* 9(12), e114018.

Larsen, S., Lundby, A. M., Dandanell, S., Oberholzer, L., Keiser, S., Andersen, A. B., Haider, T. and Lundby, C. (2018) 'Four days of bed rest increases intrinsic mitochondrial respiratory capacity in young healthy males', *Physiol Rep,* 6(18), e13793.

Laughlin, M. H. and Roseguini, B. (2008) 'Mechanisms for exercise training-induced increases in skeletal muscle blood flow capacity: differences with interval sprint training versus aerobic endurance training', *J Physiol Pharmacol,* 59 Suppl 7(Suppl 7), 71-88.

Lauritzen, H. P. M. M., Brandauer, J., Schjerling, P., Koh, H.-J., Treebak, J. T., Hirshman, M. F., Galbo, H. and Goodyear, L. J. (2013) 'Contraction and AICAR Stimulate IL-6 Vesicle Depletion From Skeletal Muscle Fibers In Vivo', *Diabetes,* 62(9), 3081-3092.

Laursen, M., Bublitz, M., Moncoq, K., Olesen, C., Møller, J. V., Young, H. S., Nissen, P. and Morth, J. P. (2009) 'Cyclopiazonic acid is complexed to a divalent metal ion when bound to the sarcoplasmic reticulum Ca2+-ATPase', *J Biol Chem,* 284(20), 13513-13518.

Laussel, C. and Léon, S. (2020) 'Cellular toxicity of the metabolic inhibitor 2-deoxyglucose and associated resistance mechanisms', *Biochemical Pharmacology,* 182, 114213.

Laussel, C., Albanèse, V., García-Rodríguez, F. J., Ballin, A., Defenouillère, Q. and Léon, S. (2022) '2-deoxyglucose transiently inhibits yeast AMPK signalling and

triggers glucose transporter endocytosis, potentiating the drug toxicity', *PLoS Genet,* 18(8), e1010169.

Lautaoja, J. H., M. O'Connell, T., Mäntyselkä, S., Peräkylä, J., Kainulainen, H., Pekkala, S., Permi, P. and Hulmi, J. J. (2021) 'Higher glucose availability augments the metabolic responses of the C2C12 myotubes to exercise-like electrical pulse stimulation', American Journal of Physiology-Endocrinology and Metabolism, 321(2), E229-E245.

Lautaoja, J. H., T, M. O. C., Mäntyselkä, S., Peräkylä, J., Kainulainen, H., Pekkala, S., Permi, P. and Hulmi, J. J. (2021) 'Higher glucose availability augments the metabolic responses of the C2C12 myotubes to exercise-like electrical pulse stimulation', *Am J Physiol Endocrinol Metab,* 321(2), E229-e245.

Lautaoja, J. H., T, M. O. C., Mäntyselkä, S., Peräkylä, J., Kainulainen, H., Pekkala, S., Permi, P. and Hulmi, J. J. (2021) 'Higher glucose availability augments the metabolic responses of the C2C12 myotubes to exercise-like electrical pulse stimulation', *Am J Physiol Endocrinol Metab,* 321(2), E229-e245.

Lautaoja, J. H., Turner, D. C., Sharples, A. P., Kivelä, R., Pekkala, S., Hulmi, J. J. and Ylä-Outinen, L. (2023) 'Mimicking exercise in vitro: effects of myotube contractions and mechanical stretch on omics', *American Journal of Physiology-Cell Physiology,* 324(4), C886-C892.

Lavie, C. J., Ozemek, C., Carbone, S., Katzmarzyk, P. T. and Blair, S. N. (2019) 'Sedentary Behavior, Exercise, and Cardiovascular Health', *Circulation Research,* 124(5), 799-815.

Lebovitz, H. E. (2011) 'Insulin: Potential Negative Consequences of Early Routine Use in Patients With Type 2 Diabetes', *Diabetes Care,* 34(Supplement 2), S225.

Lee, B., Kim, S. K., Shin, Y. J., Son, Y. H., Yang, J. W., Lee, S.-M., Yang, Y. R., Lee, K.-P. and Kwon, K.-S. (2022) 'Genome-wide analysis of a cellular exercise model based on electrical pulse stimulation', *Scientific Reports,* 12(1), 21251.

Lee, J. H. and Jun, H.-S. (2019) 'Role of Myokines in Regulating Skeletal Muscle Mass and Function', *Frontiers in Physiology,* 10(42).

Lee, S. J., Lehar, A., Meir, J. U., Koch, C., Morgan, A., Warren, L. E., Rydzik, R., Youngstrom, D. W., Chandok, H., George, J., Gogain, J., Michaud, M., Stoklasek, T. A., Liu, Y. and Germain-Lee, E. L. (2020) 'Targeting myostatin/activin A protects against skeletal muscle and bone loss during spaceflight', *Proc Natl Acad Sci U S A,* 117(38), 23942-23951.

Leermakers, P. A., Kneppers, A. E. M., Schols, A., Kelders, M., de Theije, C. C., Verdijk, L. B., van Loon, L. J. C., Langen, R. C. J. and Gosker, H. R. (2019) 'Skeletal muscle unloading results in increased mitophagy and decreased mitochondrial biogenesis regulation', Muscle Nerve, 60(6), 769-778.

Leguy, C. A. D., Delfos, R., Pourquie, M. J. B. M., Poelma, C., Westerweel, J. and van Loon, J. J. W. A. (2017) 'Fluid dynamics during Random Positioning Machine micro-gravity experiments', *Advances in Space Research,* 59(12), 3045-3057.

Lehrskov, L. L. and Christensen, R. H. (2019) 'The role of interleukin-6 in glucose homeostasis and lipid metabolism', *Seminars in Immunopathology,* 41(4), 491-499.

Leprivier, G. and Rotblat, B. (2020) 'How does mTOR sense glucose starvation? AMPK is the usual suspect', *Cell Death Discov,* 6, 27.

Lewis, S. E., Anderson, P. and Goldspink, D. F. (1982) 'The effects of calcium on protein turnover in skeletal muscles of the rat', Biochem J, 204(1), 257-64.

Li, W. Y., Li, X. Y., Tian, Y. H., Chen, X. R., Zhou, J., Zhu, B. Y., Xi, H. R., Gao, Y. H., Xian, C. J. and Chen, K. M. (2018) 'Pulsed electromagnetic fields prevented the decrease of bone formation in hindlimb-suspended rats by activating sAC/cAMP/PKA/CREB signalling pathway', *Bioelectromagnetics,* 39(8), 569-584.

Li, Y. P., Chen, Y., Li, A. S. and Reid, M. B. (2003) 'Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes', *Am J Physiol Cell Physiol,* 285(4), C806-12.

Li, Z., Yue, Y., Hu, F., Zhang, C., Ma, X., Li, N., Qiu, L., Fu, M., Chen, L., Yao, Z., Bilan, P. J., Klip, A. and Niu, W. (2017) 'Electrical pulse stimulation induces GLUT4 translocation in C2C12 myotubes that depends on Rab8A, Rab13, and Rab14', *American Journal of Physiology-Endocrinology and Metabolism,* 314(5), E478-E493.

Li, Z., Yue, Y., Hu, F., Zhang, C., Ma, X., Li, N., Qiu, L., Fu, M., Chen, L., Yao, Z., Bilan, P. J., Klip, A. and Niu, W. (2017) 'Electrical pulse stimulation induces GLUT4 translocation in C2C12 myotubes that depends on Rab8A, Rab13, and Rab14', American Journal of Physiology-Endocrinology and Metabolism, 314(5), E478-E493.

Lian, D., Chen, M. M., Wu, H., Deng, S. and Hu, X. (2022) 'The Role of Oxidative Stress in Skeletal Muscle Myogenesis and Muscle Disease', Antioxidants (Basel), 11(4).

Liang, H. and Ward, W. F. (2006) 'PGC-1α: a key regulator of energy metabolism', *Advances in Physiology Education,* 30(4), 145-151.

Liao, J., Li, Y., Zeng, F. and Wu, Y. (2015) 'Regulation of mTOR Pathway in Exercise-induced Cardiac Hypertrophy', *Int J Sports Med,* 36(5), 343-50.

Litwack, G., 2018. Glycolysis and Gluconeogenesis. In: G. Litwack, ed., *Human Biochemistry,* 1st ed. [online] Elsevier, pp.183-198. Available at: <https://www.sciencedirect.com/science/article/pii/B9780123838643000089?via%3Dihub> [Accessed 10 May 2020].

Liu, L., Yang, H., Kuang, S.-J., Zhang, L., Zhou, M.-Y., Zeng, P., Zhang, M.-Z., Rao, F., Zhou, Z.-L. and Deng, C.-Y. (2023) 'Contribution of calcium dysregulation to impaired coronary artery contraction in Zucker diabetic fatty rats', *Clinical and Experimental Pharmacology and Physiology,* 50(2), 158-168.

Liu, M., Wang, L., Li, X., Wu, Y., Yin, F. and Liu, J. (2020) 'Trilobatin ameliorates insulin resistance through IRS-AKT-GLUT4 signalling pathway in C2C12 myotubes and ob/ob mice', *Chinese Medicine,* 15(1), 110.

Long, D. E., Kosmac, K., Dungan, C. M., Bamman, M. M., Peterson, C. A. and Kern, P. A. (2022) 'Potential Benefits of Combined Statin and Metformin Therapy on Resistance Training Response in Older Individuals', *Front Physiol,* 13, 872745.

Loubiere, C., Clavel, S., Gilleron, J., Harisseh, R., Fauconnier, J., Ben-Sahra, I., Kaminski, L., Laurent, K., Herkenne, S., Lacas-Gervais, S., Ambrosetti, D., Alcor, D., Rocchi, S., Cormont, M., Michiels, J. F., Mari, B., Mazure, N. M., Scorrano, L., Lacampagne, A., Gharib, A., Tanti, J. F. and Bost, F. (2017) 'The energy disruptor metformin targets mitochondrial integrity via modification of calcium flux in cancer cells', *Sci Rep,* 7(1), 5040.

Louis, F., Bouleftour, W., Rattner, A., Linossier, M. T., Vico, L. and Guignandon, A. (2017) 'RhoGTPase stimulation is associated with strontium chloride treatment to counter simulated microgravity-induced changes in multipotent cell commitment', *Npj Microgravity,* 3, 12.

Lu, Z. and Xu, S. (2006) 'ERK1/2 MAP kinases in cell survival and apoptosis', IUBMB Life, 58(11), 621-31.

Luciano, T. F., Marques, S. O., Pieri, B. L., de Souza, D. R., Araújo, L. V., Nesi, R. T., Scheffer, D. L., Comin, V. H., Pinho, R. A., Muller, A. P. and de Souza, C. T. (2017) 'Responses of skeletal muscle hypertrophy in Wistar rats to different resistance exercise models', *Physiol Res,* 66(2), 317-323.

Luo, W., Ai, L., Wang, B.-f. and Zhou, Y. (2019) 'High glucose inhibits myogenesis and induces insulin resistance by down-regulating AKT signalling', *Biomedicine & Pharmacotherapy,* 120, 109498.

Luo, W., Ai, L., Wang, B.-f. and Zhou, Y. (2019) 'High glucose inhibits myogenesis and induces insulin resistance by down-regulating AKT signalling', Biomedicine & Pharmacotherapy, 120, 109498.

Mabilleau, G., Pereira, M. and Chenu, C. (2018) 'Novel skeletal effects of glucagon-like peptide-1 (GLP-1) receptor agonists', *Journal of Endocrinology,* 236(1), R29-R42.

MacDougall, J. D., Gibala, M. J., Tarnopolsky, M. A., MacDonald, J. R., Interisano, S. A. and Yarasheski, K. E. (1995) 'The Time Course for Elevated Muscle Protein

Synthesis Following Heavy Resistance Exercise', *Canadian Journal of Applied Physiology,* 20(4), 480-486.

Mackenzie, R. W. and Elliott, B. T. (2014) 'Akt/PKB activation and insulin signalling: a novel insulin signalling pathway in the treatment of type 2 diabetes', *Diabetes Metab Syndr Obes,* 7, 55-64.

Maffiuletti, N. A., Green, D. A., Vaz, M. A. and Dirks, M. L. (2019) 'Neuromuscular Electrical Stimulation as a Potential Countermeasure for Skeletal Muscle Atrophy and Weakness During Human Spaceflight', *Frontiers in Physiology,* 10(1031).

Manabe, Y., Miyatake, S., Takagi, M., Nakamura, M., Okeda, A., Nakano, T., Hirshman, M. F., Goodyear, L. J. and Fujii, N. L. (2013) 'Characterization of an Acute Muscle Contraction Model Using Cultured C2C12 Myotubes', *PLOS ONE,* 7(12), e52592.

Manabe, Y., Miyatake, S., Takagi, M., Nakamura, M., Okeda, A., Nakano, T., Hirshman, M. F., Goodyear, L. J. and Fujii, N. L. (2013) 'Characterization of an Acute Muscle Contraction Model Using Cultured C2C12 Myotubes', PLOS ONE, 7(12), e52592.

Manabe, Y., Takagi, M., Nakamura-Yamada, M., Goto-Inoue, N., Taoka, M., Isobe, T. and Fujii, N. L. (2014) 'Redox proteins are constitutively secreted by skeletal muscle', *The Journal of Physiological Sciences,* 64(6), 401-409.

Mangnall, D., Bruce, C. and Fraser, R. (1993) 'Insulin-stimulated glucose uptake in C2C12 myoblasts', *Biochemical Society transactions,* 21, 438S.

Marchant, E. D., Corbin, W. M., Eyre, C. L. and Marchant, N. D. 'Unlike a high-fat diet model, mitochondrial ROS production does not appear to contribute to bed rest-induced insulin resistance', *Journal of Physiology-London,* 2.

Marcus, R. L., Addison, O., Kidde, J. P., Dibble, L. E. and Lastayo, P. C. (2010) 'Skeletal muscle fat infiltration: impact of age, inactivity, and exercise', *J Nutr Health Aging,* 14(5), 362-6.

Mariano, A. C., Alexandre, G. M., Silva, L. C., Romeiro, A., Cameron, L. C., Chen, Y., Chase, P. B. and Sorenson, M. M. (2001) 'Dimethyl sulphoxide enhances the effects of P(i) in myofibrils and inhibits the activity of rabbit skeletal muscle contractile proteins', Biochem J, 358(Pt 3), 627-36.

Marius, P., Guerra, M. T., Nathanson, M. H., Ehrlich, B. E. and Leite, M. F. (2006) 'Calcium release from ryanodine receptors in the nucleoplasmic reticulum', *Cell Calcium,* 39(1), 65-73.

Marotta, M., Bragós, R. and Gómez-Foix, A. M. (2004) 'Design and performance of an electrical stimulator for long-term contraction of cultured muscle cells', BioTechniques, 36(1), 68-73.

Martherus, R. S. R. M., Vanherle, S. J. V., Timmer, E. D. J., Zeijlemaker, V. A., Broers, J. L., Smeets, H. J., Geraedts, J. P. and Ayoubi, T. A. Y. (2010) 'Electrical signals affect the cardiomyocyte transcriptome independently of contraction', *Physiological Genomics,* 42A(4), 283-289.

Masson, S. W. C., Woodhead, J. S. T., D'Souza, R. F., Broome, S. C., MacRae, C., Cho, H. C., Atiola, R. D., Futi, T., Dent, J. R., Shepherd, P. R. and Merry, T. L. (2021) 'β-Catenin is required for optimal exercise- and contraction-stimulated skeletal muscle glucose uptake', *The Journal of Physiology,* 599(16), 3897-3912.
Masson, S. W. C., Woodhead, J. S. T., D'Souza, R. F., Broome, S. C., MacRae, C., Cho, H. C., Atiola, R. D., Futi, T., Dent, J. R., Shepherd, P. R. and Merry, T. L. (2021) 'β-Catenin is required for optimal exercise- and contraction-stimulated skeletal muscle glucose uptake', *The Journal of Physiology,* 599(16), 3897-3912.
Massry, S. G. and Smogorzewski, M. (1997) 'Role of elevated cytosolic calcium in the pathogenesis of complications in diabetes mellitus', *Miner Electrolyte Metab,* 23(3-6), 253-60.

Mathew, A. V., Jaiswal, M., Ang, L., Michailidis, G., Pennathur, S. and Pop-Busui, R. (2019) 'Impaired Amino Acid and TCA Metabolism and Cardiovascular Autonomic Neuropathy Progression in Type 1 Diabetes', *Diabetes,* 68(10), 2035.

Matsunaga, T., Shimada, Y. and Sato, K. (1999) 'Muscle fatigue from intermittent stimulation with low and high frequency electrical pulses', *Arch Phys Med Rehabil,* 80(1), 48-53.

Matuszczak, Y., Arbogast, S. and Reid, M. B. (2004) 'Allopurinol mitigates muscle contractile dysfunction caused by hindlimb unloading in mice', *Aviation Space and Environmental Medicine,* 75(7), 581-588.

McCarthy, J. J. and Esser, K. A. (2010) 'Anabolic and catabolic pathways regulating skeletal muscle mass', *Curr Opin Clin Nutr Metab Care,* 13(3), 230-5.

McCracken, E., Monaghan, M. and Sreenivasan, S. (2018) 'Pathophysiology of the metabolic syndrome', *Clinics in Dermatology,* 36(1), 14-20.

McKenna, C. F. and Fry, C. S. (2017) 'Altered satellite cell dynamics accompany skeletal muscle atrophy during chronic illness, disuse, and aging', *Curr Opin Clin Nutr Metab Care,* 20(6), 447-452.

McLeod, J. C., Stokes, T. and Phillips, S. M. (2019) 'Resistance Exercise Training as a Primary Countermeasure to Age-Related Chronic Disease', *Frontiers in Physiology,* 10, 11.

McPherron, A. C., Guo, T., Bond, N. D. and Gavrilova, O. (2013) 'Increasing muscle mass to improve metabolism', *Adipocyte,* 2(2), 92-8.

Mehta, P. and Bhayani, D. (2017) 'Impact of space environment on stability of medicines: Challenges and prospects', *Journal of Pharmaceutical and Biomedical Analysis,* 136, 111-119.

Melick, C. H. and Jewell, J. L. (2020) 'Regulation of mTORC1 by Upstream Stimuli', *Genes,* 11(9), 989.

Melkonian EA, Schury MP. Biochemistry, Anaerobic Glycolysis. [Updated 2019 Aug 21]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK546695/

Melouane, A., Yoshioka, M., Kanzaki, M. and St-Amand, J. (2019) 'Sparc, an EPS-induced gene, modulates the extracellular matrix and mitochondrial function via ILK/AMPK pathways in C2C12 cells', *Life Sciences,* 229, 277-287.

Melzer, K. (2011) 'Carbohydrate and fat utilization during rest and physical activity', *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism,* 6(2), e45-e52.

Même, W., Huchet-Cadiou, C. and Léoty, C. (1998) 'Cyclopiazonic acid-induced changes in the contraction and Ca2+ transient of frog fast-twitch skeletal muscle', American Journal of Physiology-Cell Physiology, 274(1), C253-C261.

Merz, K. E. and Thurmond, D. C. (2020) 'Role of Skeletal Muscle in Insulin Resistance and Glucose Uptake', *Compr Physiol,* 10(3), 785-809.

Mettler, J. A., Magee, D. M. and Doucet, B. M. (2018) 'High-Frequency Neuromuscular Electrical Stimulation Increases Anabolic Signaling', *Med Sci Sports Exerc,* 50(8), 1540-1548.

Meyler, S., Bottoms, L. and Muniz-Pumares, D. (2021) 'Biological and methodological factors affecting response variability to endurance training and the influence of exercise intensity prescription', *Experimental Physiology,* 106(7), 1410-1424.

Mikines, K. J., Richter, E. A., Dela, F. and Galbo, H. (1991) 'Seven days of bed rest decrease insulin action on glucose uptake in leg and whole body', *JAppl Physiol (1985),* 70(3), 1245-54.

Miller, B. F., Baehr, L. M., Musci, R. V., Reid, J. J., Peelor, F. F., 3rd, Hamilton, K. L. and Bodine, S. C. (2019) 'Muscle-specific changes in protein synthesis with aging and reloading after disuse atrophy', *J Cachexia Sarcopenia Muscle,* 10(6), 1195-1209.

Mirzoev, T. M. and Shenkman, B. S. (2018) 'Regulation of Protein Synthesis in Inactivated Skeletal Muscle: Signal Inputs, Protein Kinase Cascades, and Ribosome Biogenesis', *Biochemistry (Moscow),* 83(11), 1299-1317.

Mirzoev, T. M., Paramonova, II, Rozhkov, S. V., Kalashnikova, E. P., Belova, S. P., Tyganov, S. A., Vilchinskaya, N. A. and Shenkman, B. S. (2023) 'Metformin Pre-Treatment as a Means of Mitigating Disuse-Induced Rat Soleus Muscle Wasting', *Curr Issues Mol Biol,* 45(4), 3068-3086.

Misquitta, N. S., Ravel-Chapuis, A. and Jasmin, B. J. (2023) 'Combinatorial treatment with exercise and AICAR potentiates the rescue of myotonic dystrophy type 1 mouse muscles in a sex-specific manner', *Human Molecular Genetics,* 32(4), 551-566.

Mitchell, C. J., Churchward-Venne, T. A., West, D. W. D., Burd, N. A., Breen, L., Baker, S. K. and Phillips, S. M. (2012) 'Resistance exercise load does not determine training-mediated hypertrophic gains in young men', *Journal of Applied Physiology,* 113(1), 71-77.

Moes, M. J. A., Gielen, J. C., Bleichrodt, R.-J., van Loon, J. J. W. A., Christianen, P. C. M. and Boonstra, J. (2011) 'Simulation of Microgravity by Magnetic Levitation and Random Positioning: Effect on Human A431 Cell Morphology', *Microgravity Science and Technology,* 23(2), 249-261.

Møller, A. B., Vendelbo, M. H., Schjerling, P., Couppé, C., Møller, N., Kjær, M., Hansen, M. and Jessen, N. (2019) 'Immobilization Decreases FOXO3a Phosphorylation and Increases Autophagy-Related Gene and Protein Expression in Human Skeletal Muscle', Frontiers in Physiology, 10.

Moncoq, K., Trieber, C. A. and Young, H. S. (2007) 'The molecular basis for cyclopiazonic acid inhibition of the sarcoplasmic reticulum calcium pump', J Biol Chem, 282(13), 9748-9757.

Monteiro, R. and Azevedo, I. (2010) 'Chronic inflammation in obesity and the metabolic syndrome', *Mediators Inflamm,* 2010.

Mooradian, A. D. and Haas, M. J. (2021) 'Reduced cellular glucose transport confers natural protection against dextrose-induced superoxide generation and endoplasmic reticulum stress in domestic hen', *Physiological Reports,* 9(7), e14816.

Moreno-Cabañas, A., Morales-Palomo, F., Alvarez-Jimenez, L., Ortega, J. F. and Mora-Rodriguez, R. (2022) 'Effects of chronic metformin treatment on training adaptations in men and women with hyperglycemia: A prospective study', *Obesity (Silver Spring),* 30(6), 1219-1230.

Morgan, J. and Partridge, T. (2020) 'Skeletal muscle in health and disease', *Disease Models & Mechanisms,* 13(2), dmm042192.

Morley, J. E. (2016) 'Pharmacologic Options for the Treatment of Sarcopenia', *Calcif Tissue Int,* 98(4), 319-33.

Mounier, R., Lantier, L., Leclerc, J., Sotiropoulos, A., Foretz, M. and Viollet, B. (2011) 'Antagonistic control of muscle cell size by AMPK and mTORC1', *Cell Cycle,* 10(16), 2640-6.

Mu, J., Brozinick, J. T., Valladares, O., Bucan, M. and Birnbaum, M. J. (2001) 'A Role for AMP-Activated Protein Kinase in Contraction- and Hypoxia-Regulated Glucose Transport in Skeletal Muscle', *Molecular Cell,* 7(5), 1085-1094.

Mueckler, M. (1994) 'FACILITATIVE GLUCOSE TRANSPORTERS', *European Journal of Biochemistry,* 219(3), 713-725.

Mukund, K. and Subramaniam, S. (2020) 'Skeletal muscle: A review of molecular structure and function, in health and disease', *WIREs Systems Biology and Medicine,* 12(1), e1462.

Muniyappa, R., Montagnani, M., Koh, K. K. and Quon, M. J. (2007) 'Cardiovascular Actions of Insulin', *Endocrine Reviews,* 28(5), 463-491.

Murata, A., Akiyama, H., Honda, H. and Shimizu, K. (2023) 'Electrical pulse stimulation-induced tetanic exercise simulation increases the secretion of extracellular vesicles from C2C12 myotubes', *Biochemical and Biophysical Research Communications,* 672, 177-184.

Murray, B. and Rosenbloom, C. (2018a) 'Fundamentals of glycogen metabolism for coaches and athletes', *Nutr Rev,* 76(4), 243-259.

Murray, B. and Rosenbloom, C. (2018b) 'Fundamentals of glycogen metabolism for coaches and athletes', *Nutr Rev,* 76(4), 243-259.

Murray, R. K., Rodwell, V. W., Bender, D., Botham, K. M., Weil, P. A. and Kennelly, P. J. (2009) *Harper's Illustrated Biochemistry, 28th Edition,* McGraw-Hill Education.

Musa, J., Orth, M. F., Dallmayer, M., Baldauf, M., Pardo, C., Rotblat, B., Kirchner, T., Leprivier, G. and Grünewald, T. G. P. (2016) 'Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1): a master regulator of mRNA translation involved in tumorigenesis', *Oncogene,* 35(36), 4675-4688.

Musacchia, X. J., Steffen, J. M. and Fell, R. D. (1988) 'Disuse Atrophy of Skeletal Muscle: Animal Models', *Exercise and Sport Sciences Reviews,* 16(1).

Muscogiuri, G., Salmon, A. B., Aguayo-Mazzucato, C., Li, M., Balas, B., Guardado-Mendoza, R., Giaccari, A., Reddick, R. L., Reyna, S. M., Weir, G., Defronzo, R. A., Van Remmen, H. and Musi, N. (2013) 'Genetic disruption of SOD1 gene causes glucose intolerance and impairs β-cell function', *Diabetes,* 62(12), 4201-7.

Musi, N., Hirshman, M. F., Nygren, J., Svanfeldt, M., Bavenholm, P., Rooyackers, O., Zhou, G., Williamson, J. M., Ljunqvist, O., Efendic, S., Moller, D. E., Thorell, A. and Goodyear, L. J. (2002) 'Metformin Increases AMP-Activated Protein Kinase Activity in Skeletal Muscle of Subjects With Type 2 Diabetes', *Diabetes,* 51(7), 2074-2081.

Muslikhov, E. R., Sukhanova, I. F. and Avdonin, P. V. (2014) 'Arachidonic acid activates release of calcium ions from reticulum via ryanodine receptor channels in C2C12 skeletal myotubes', *Biochemistry (Moscow),* 79(5), 435-439.

Nakanishi, R., Tanaka, M., Maeshige, N., Kondo, H., Roy, R. R. and Fujino, H. (2021) 'Nucleoprotein-enriched diet enhances protein synthesis pathway and satellite cell activation via ERK1/2 phosphorylation in unloaded rat muscles', *Experimental Physiology,* 106(7), 1587-1596.

Narici, M., De Vito, G., Franchi, M., Paoli, A., Moro, T., Marcolin, G., Grassi, B., Baldassarre, G., Zuccarelli, L., Biolo, G., di Girolamo, F. G., Fiotti, N., Dela, F., Greenhaff, P. and Maganaris, C. (2020) 'Impact of sedentarism due to the COVID-19 home confinement on neuromuscular, cardiovascular and metabolic health: Physiological and pathophysiological implications and recommendations for physical and nutritional countermeasures', *European Journal of Sport Science*, 1-22.

Narkar, V. A., Downes, M., Yu, R. T., Embler, E., Wang, Y. X., Banayo, E., Mihaylova, M. M., Nelson, M. C., Zou, Y., Juguilon, H., Kang, H., Shaw, R. J. and Evans, R. M. (2008) 'AMPK and PPARdelta agonists are exercise mimetics', *Cell,* 134(3), 405-15.

NASA (1984) 'Final Report Phase IV: Research Opportunities in Muscle Atrophy', [Online] Available at: https://ntrs.nasa.gov/archive/nasa/casi.ntrs.nasa.gov/19840019348.pdf. Accessed 01/06/2020

NASA (2018) Rodent research 6 (SpaceX-13) [Online]. Available at: https://www.nasa.gov/ames/research/space-biosciences/rodent-research-6-spacex-13. Accessed 20/01/2020

NASA (2020) NASA Selects Proposals to Study Adaptation and Response for Astronaut Missions to Moon, Mars [Online]. Available at https://www.nasa.gov/feature/nasa-selects-proposals-to-study-adaptation-and-response-for-astronaut-missions-to-moon-mars Accessed 01/06/2020

Nedachi, T., Fujita, H. and Kanzaki, M. (2008) 'Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle', *Am J Physiol Endocrinol Metab,* 295(5), E1191-204.

Nedachi, T., Fujita, H. and Kanzaki, M. (2008) 'Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle', *Am J Physiol Endocrinol Metab,* 295(5), E1191-204.

Negoita, F., Addinsall, A. B., Hellberg, K., Bringas, C. F., Hafen, P. S., Sermersheim, T. J., Agerholm, M., Lewis, C. T. A., Ahwazi, D., Ling, N. X. Y., Larsen, J. K., Deshmukh, A. S., Hossain, M. A., Oakhill, J. S., Ochala, J., Brault, J. J., Sankar, U., Drewry, D. H., Scott, J. W., Witczak, C. A. and Sakamoto, K.

(2023) 'CaMKK2 is not involved in contraction-stimulated AMPK activation and glucose uptake in skeletal muscle', *Molecular Metabolism*, 101761.

Negre-Salvayre, A., Salvayre, R., Augé, N., Pamplona, R. and Portero-Otín, M. (2009) 'Hyperglycemia and glycation in diabetic complications', *Antioxid Redox Signal,* 11(12), 3071-109.

Nemirovskaya, T. L. and Sharlo, K. A. (2022) 'Roles of ATP and SERCA in the Regulation of Calcium Turnover in Unloaded Skeletal Muscles: Current View and Future Directions', Int J Mol Sci, 23(13).

Nikolic, N., Bakke, S. S., Kase, E. T., Rudberg, I., Flo Halle, I., Rustan, A. C., Thoresen, G. H. and Aas, V. (2012) 'Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise', *PLoS One,* 7(3), e33203.

Nikolić, N., Skaret Bakke, S., Tranheim Kase, E., Rudberg, I., Flo Halle, I., Rustan, A. C., Thoresen, G. H. and Aas, V. (2012) 'Electrical Pulse Stimulation of Cultured Human Skeletal Muscle Cells as an In Vitro Model of Exercise', PLOS ONE, 7(3), e33203.

Nintou, E., Karligiotou, E., Vliora, M., Ioannou, L. G. and Flouris, A. D. (2022) 'Characteristics of the Protocols Used in Electrical Pulse Stimulation of Cultured Cells for Mimicking In Vivo Exercise: A Systematic Review, Meta-Analysis, and Meta-Regression', *International Journal of Molecular Sciences,* 23(21).

Norred, W. P., Morrissey, R. E., Riley, R. T., Cole, R. J. and Dorner, J. W. (1985) 'Distribution, excretion and skeletal muscle effects of the mycotoxin [14C]cyclopiazonic acid in rats', Food Chem Toxicol, 23(12), 1069-76.

Ogasawara, R., Sato, K., Matsutani, K., Nakazato, K. and Fujita, S. (2014) 'The order of concurrent endurance and resistance exercise modifies mTOR signalling and protein synthesis in rat skeletal muscle', *American Journal of Physiology-Endocrinology and Metabolism,* 306(10), E1155-E1162.

Ohno, Y., Ando, K., Ito, T., Suda, Y., Matsui, Y., Oyama, A., Kaneko, H., Yokoyama, S., Egawa, T. and Goto, K. (2019) 'Lactate Stimulates a Potential for Hypertrophy and Regeneration of Mouse Skeletal Muscle', *Nutrients,* 11(4).

Ojuka, E. O., Jones, T. E., Nolte, L. A., Chen, M., Wamhoff, B. R., Sturek, M. and Holloszy, J. O. (2002) 'Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca2+', *American Journal of Physiology-Endocrinology and Metabolism,* 282(5), E1008-E1013.

Olson, A. L., Trumbly, A. R. and Gibson, G. V. (2001) 'Insulin-mediated GLUT4 Translocation Is Dependent on the Microtubule Network *', *Journal of Biological Chemistry,* 276(14), 10706-10714.

Olthoff, J. T., Lindsay, A., Abo-Zahrah, R., Baltgalvis, K. A., Patrinostro, X., Belanto, J. J., Yu, D. Y., Perrin, B. J., Garry, D. J., Rodney, G. G., Lowe, D. A. and

Ervasti, J. M. (2018) 'Loss of peroxiredoxin-2 exacerbates eccentric contraction-induced force loss in dystrophin-deficient muscle', *Nat Commun,* 9(1), 5104.

O'Neill, H. M. (2013a) 'AMPK and Exercise: Glucose Uptake and Insulin Sensitivity', *Diabetes Metab J,* 37(1), 1-21.

O'Neill, H. M. (2013b) 'AMPK and Exercise: Glucose Uptake and Insulin Sensitivity', *Diabetes Metab J,* 37(1), 1-21.

Ong, P. S., Wang, L. Z., Dai, X., Tseng, S. H., Loo, S. J. and Sethi, G. (2016) 'Judicious Toggling of mTOR Activity to Combat Insulin Resistance and Cancer: Current Evidence and Perspectives', *Front Pharmacol,* 7, 395.

Oresic, M., Simell, S., Sysi-Aho, M., Nanto-Salonen, K., Seppanen-Laakso, T., Parikka, V., Katajamaa, M., Hekkala, A., Mattila, I., Keskinen, P., Yetukuri, L., Reinikainen, A., Lahde, J., Suortti, T., Hakalax, J., Simell, T., Hyoty, H., Veijola, R., Ilonen, J., Lahesmaa, R., Knip, M. and Simell, O. (2008) 'Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes', *J Exp Med,* 205(13), 2975-84.

Orfanos, Z., Gödderz, M. P., Soroka, E., Gödderz, T., Rumyantseva, A., van der Ven, P. F., Hawke, T. J. and Fürst, D. O. (2016) 'Breaking sarcomeres by in vitro exercise', *Sci Rep,* 6, 19614.

Ørtenblad, N., Westerblad, H. and Nielsen, J. (2013) 'Muscle glycogen stores and fatigue', *The Journal of Physiology,* 591(18), 4405-4413.

Orzechowski, A., Grzelkowska, K., Karlik, W., Tomasz and Motyl (2002) Effect of Quercetin and DMSO on Skeletal Myogenesis from C 2 C 12 Skeletal Muscle Cells with Special Reference to PKB / Akt Activity , Myogenin and Bcl-2 Expression, translated by.

Ostman, C., Jewiss, D., King, N. and Smart, N. A. (2018) 'Clinical outcomes to exercise training in type 1 diabetes: A systematic review and meta-analysis', *Diabetes Research and Clinical Practice,* 139, 380-391.

Ostry, V., Toman, J., Grosse, Y. and Malir, F. (2018) 'Cyclopiazonic acid: 50 th anniversary of its discovery', World Mycotoxin Journal, 11, 135-148.

Pajak, B., Siwiak, E., Sołtyka, M., Priebe, A., Zieliński, R., Fokt, I., Ziemniak, M., Jaśkiewicz, A., Borowski, R., Domoradzki, T. and Priebe, W. (2019) '2-Deoxy-d-Glucose and Its Analogs: From Diagnostic to Therapeutic Agents', *Int J Mol Sci,* 21(1).

Paneni, F., Beckman, J. A., Creager, M. A. and Cosentino, F. (2013) 'Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I', *Eur Heart J,* 34(31), 2436-43.

Park, K. H. (2015) 'Mechanisms of Muscle Denervation in Aging: Insights from a Mouse Model of Amyotrophic Lateral Sclerosis', *Aging Dis,* 6(5), 380-9.

Park, S., Scheffler, T. L. and Gerrard, D. E. (2011) 'Chronic high cytosolic calcium decreases AICAR-induced AMPK activity via calcium/calmodulin activated protein kinase II signalling cascade', *Cell Calcium,* 50(1), 73-83.

Park, S., Scheffler, T. L., Rossie, S. S. and Gerrard, D. E. (2013) 'AMPK activity is regulated by calcium-mediated protein phosphatase 2A activity', *Cell Calcium,* 53(3), 217-23.

Parry, S. M. and Puthucheary, Z. A. (2015) 'The impact of extended bed rest on the musculoskeletal system in the critical care environment', Extrem Physiol Med, 4, 16.

Parry, S. M. and Puthucheary, Z. A. (2015) 'The impact of extended bed rest on the musculoskeletal system in the critical care environment', Extreme Physiology & Medicine, 4(1), 16.

Pedrosa, M. B., Barbosa, S., Vitorino, R., Ferreira, R., Moreira-Gonçalves, D. and Santos, L. L. (2023) 'Chemotherapy-Induced Molecular Changes in Skeletal Muscle', *Biomedicines,* 11(3).

Pernicova, I. and Korbonits, M. (2014) 'Metformin--mode of action and clinical implications for diabetes and cancer', *Nat Rev Endocrinol,* 10(3), 143-56.

Petersen, M. C. and Shulman, G. I. (2018) 'Mechanisms of Insulin Action and Insulin Resistance', *Physiol Rev,* 98(4), 2133-2223.

Petridou, A., Siopi, A. and Mougios, V. (2019) 'Exercise in the management of obesity', *Metabolism,* 92, 163-169.

Pilmark, N. S., Oberholzer, L., Halling, J. F., Kristensen, J. M., Bønding, C. P., Elkjær, I., Lyngbæk, M., Elster, G., Siebenmann, C., Holm, N. F. R., Birk, J. B., Larsen, E. L., Lundby, A. M., Wojtaszewski, J., Pilegaard, H., Poulsen, H. E., Pedersen, B. K., Hansen, K. B. and Karstoft, K. (2022) 'Skeletal muscle adaptations to exercise are not influenced by metformin treatment in humans: secondary analyses of 2 randomized, clinical trials', *Appl Physiol Nutr Metab,* 47(3), 309-320.

Plotkin, D. L., Roberts, M. D., Haun, C. T. and Schoenfeld, B. J. (2021) 'Muscle Fiber Type Transitions with Exercise Training: Shifting Perspectives', *Sports (Basel),* 9(9).

Polonsky, K. S., Given, B. D. and Van Cauter, E. (1988) 'Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects', *The Journal of Clinical Investigation,* 81(2), 442-448.

Powers, S. K., Smuder, A. J. and Judge, A. R. (2012) 'Oxidative stress and disuse muscle atrophy: cause or consequence?', Current Opinion in Clinical Nutrition & Metabolic Care, 15(3).

Prince, F., Corriveau, H., Hébert, R. and Winter, D. A. (1997) 'Gait in the elderly', *Gait & Posture,* 5(2), 128-135.

Prior, S. J., Goldberg, A. P., Ortmeyer, H. K., Chin, E. R., Chen, D., Blumenthal, J. B. and Ryan, A. S. (2015) 'Increased Skeletal Muscle Capillarization Independently Enhances Insulin Sensitivity in Older Adults After Exercise Training and Detraining', *Diabetes,* 64(10), 3386-95.

Qaisar, R., Pharaoh, G., Bhaskaran, S., Xu, H., Ranjit, R., Bian, J., Ahn, B., Georgescu, C., Wren, J. D. and Van Remmen, H. (2020) 'Restoration of Sarcoplasmic Reticulum Ca(2+) ATPase (SERCA) Activity Prevents Age-Related Muscle Atrophy and Weakness in Mice', *Int J Mol Sci,* 22(1).

Qin, L., Knol, M. J., Corpeleijn, E. and Stolk, R. P. (2010) 'Does physical activity modify the risk of obesity for type 2 diabetes: a review of epidemiological data', *Eur J Epidemiol,* 25(1), 5-12.

Qin, X., Jiang, B. and Zhang, Y. (2016) '4E-BP1, a multifactor regulated multifunctional protein', *Cell Cycle,* 15(6), 781-6.

Qin, X., Jiang, B. and Zhang, Y. (2016) '4E-BP1, a multifactor regulated multifunctional protein', Cell Cycle, 15(6), 781-6.

Racca, A. W., Beck, A. E., Rao, V. S., Flint, G. V., Lundy, S. D., Born, D. E., Bamshad, M. J. and Regnier, M. (2013) 'Contractility and kinetics of human fetal and human adult skeletal muscle', *J Physiol,* 591(12), 3049-61.

Radak, Z., Suzuki, K., Higuchi, M., Balogh, L., Boldogh, I. and Koltai, E. (2016) 'Physical exercise, reactive oxygen species and neuroprotection', *Free Radical Biology and Medicine,* 98, 187-196.

Radigan, K. A., Nicholson, T. T., Welch, L. C., Chi, M., Amarelle, L., Angulo, M., Shigemura, M., Shigemura, A., Runyan, C. E., Morales-Nebreda, L., Perlman, H., Ceco, E., Lecuona, E., Dada, L. A., Misharin, A. V., Mutlu, G. M., Sznajder, J. I. and Budinger, G. R. S. (2019) 'Influenza A Virus Infection Induces Muscle Wasting via IL-6 Regulation of the E3 Ubiquitin Ligase Atrogin-1', *J Immunol,* 202(2), 484-493.

Rahman, M. A., Ušaj, M., Rassier, D. E. and Månsson, A. (2018) 'Blebbistatin Effects Expose Hidden Secrets in the Force-Generating Cycle of Actin and Myosin', Biophys J, 115(2), 386-397.

Rai, B., Kaur, J. and Foing, B. H. (2012) 'Stress, workload and physiology demand during extravehicular activity: a pilot study', *N Am J Med Sci,* 4(6), 266-9.

Ralser, M., Wamelink, M. M., Struys, E. A., Joppich, C., Krobitsch, S., Jakobs, C. and Lehrach, H. (2008) 'A catabolic block does not sufficiently explain how 2-deoxy-D-glucose inhibits cell growth', *Proc Natl Acad Sci U S A,* 105(46), 17807-11.

Rassier, D. E. (2017) 'Sarcomere mechanics in striated muscles: from molecules to sarcomeres to cells', *American Journal of Physiology-Cell Physiology,* 313(2), C134-C145.

Rauscher, A., Gyimesi, M., Kovács, M. and Málnási-Csizmadia, A. (2018) 'Targeting Myosin by Blebbistatin Derivatives: Optimization and Pharmacological Potential', Trends Biochem Sci, 43(9), 700-713.

Rennie, M. J., Wackerhage, H., Spangenburg, E. E. and Booth, F. W. (2004) 'Control of the size of the human muscle mass', *Annu Rev Physiol,* 66, 799-828.

Richter, E. A. and Hargreaves, M. (2013) 'Exercise, GLUT4, and Skeletal Muscle Glucose Uptake', *Physiological Reviews,* 93(3), 993-1017.

Richter, E. A. and Ruderman, N. B. (2009) 'AMPK and the biochemistry of exercise: implications for human health and disease', *Biochem J,* 418(2), 261-75.

Rion, N., Castets, P., Lin, S., Enderle, L., Reinhard, J. R. and Rüegg, M. A. (2019) 'mTORC2 affects the maintenance of the muscle stem cell pool', *Skeletal Muscle,* 9(1), 30.

Ristow, M., Zarse, K., Oberbach, A., Kloting, N., Birringer, M., Kiehntopf, M., Stumvoll, M., Kahn, C. R. and Bluher, M. (2009) 'Antioxidants prevent health-promoting effects of physical exercise in humans', *Proc Natl Acad Sci U S A,* 106(21), 8665-70.

Rivas, D., Lessard, S. and Coffey, V. (2009) 'mTOR function in skeletal muscle: A focal point for overnutrition and exercise', *Applied physiology, nutrition, and metabolism = Physiologie appliquée, nutrition et métabolisme,* 34, 807-16.

Robergs, R. A., Ghiasvand, F. and Parker, D. (2004) 'Biochemistry of exercise-induced metabolic acidosis', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 287(3), R502-R516.

Robinson, M. M., Sather, B. K., Burney, E. R., Ehrlicher, S. E., Stierwalt, H. D., Franco, M. C. and Newsom, S. A. (2019) 'Robust intrinsic differences in mitochondrial respiration and H2O2 emission between L6 and C2C12 cells', *American Journal of Physiology-Cell Physiology,* 317(2), C339-C347.

Röckl, K. S. C., Witczak, C. A. and Goodyear, L. J. (2008) 'Signalling mechanisms in skeletal muscle: Acute responses and chronic adaptations to exercise', IUBMB Life, 60(3), 145-153.

Rodwell, V., Bender, D., Botham, K., Weil, P. and Kennelly, P., 2009. *Harper's Illustrated Biochemistry, 28Th Edition*. 28th ed. McGraw-Hill Publishing, pp.302-312.

Rolli-Derkinderen, M., Machavoine, F., Baraban, J. M., Grolleau, A., Beretta, L. and Dy, M. (2003) 'ERK and p38 inhibit the expression of 4E-BP1 repressor of translation through induction of Egr-1', *J Biol Chem,* 278(21), 18859-67.

Rosa-Caldwell, M. E., Fix, D. K., Washington, T. A. and Greene, N. P. (2019) 'Muscle alterations in the development and progression of cancer-induced muscle atrophy: a review', *Journal of Applied Physiology,* 128(1), 25-41.

Rose, A. J., Alsted, T. J., Jensen, T. E., Kobberø, J. B., Maarbjerg, S. J., Jensen, J. and Richter, E. A. (2009a) 'A Ca2+–calmodulin–eEF2K–eEF2 signalling cascade, but not AMPK, contributes to the suppression of skeletal muscle protein synthesis during contractions', The Journal of Physiology, 587(7), 1547-1563.

Rose, A. J., Alsted, T. J., Jensen, T. E., Kobberø, J. B., Maarbjerg, S. J., Jensen, J. and Richter, E. A. (2009b) 'A Ca2+–calmodulin–eEF2K–eEF2 signalling cascade, but not AMPK, contributes to the suppression of skeletal muscle protein synthesis during contractions', The Journal of Physiology, 587(7), 1547-1563.

Rosenfeld, L. (2002) 'Insulin: Discovery and Controversy', *Clinical Chemistry,* 48(12), 2270-2288.

Rowan, S. L., Rygiel, K., Purves-Smith, F. M., Solbak, N. M., Turnbull, D. M. and Hepple, R. T. (2012) 'Denervation causes fiber atrophy and myosin heavy chain co-expression in senescent skeletal muscle', *PLoS One,* 7(1), e29082.

Rozhkov, S. V., Sharlo, K. A., Shenkman, B. S. and Mirzoev, T. M. (2022) 'Inhibition of mTORC1 differentially affects ribosome biogenesis in rat soleus muscle at the early and later stages of hindlimb unloading', Archives of Biochemistry and Biophysics, 730, 109411.

Ruan, X., Cao, M., Yan, W., Jones, Y. Z., Gustafsson Å, B., Patel, H. H., Schenk, S. and Wang, S. E. (2023) 'Cancer-cell-secreted extracellular vesicles target p53 to impair mitochondrial function in muscle', *EMBO Rep*, e56464.

Rudrappa, S. S., Wilkinson, D. J., Greenhaff, P. L., Smith, K., Idris, I. and Atherton, P. J. (2016) 'Human Skeletal Muscle Disuse Atrophy: Effects on Muscle Protein Synthesis, Breakdown, and Insulin Resistance-A Qualitative Review', *Front Physiol,* 7, 361.

Rudrappa, S. S., Wilkinson, D. J., Greenhaff, P. L., Smith, K., Idris, I. and Atherton, P. J. (2016) 'Human Skeletal Muscle Disuse Atrophy: Effects on Muscle Protein Synthesis, Breakdown, and Insulin Resistance-A Qualitative Review', *Front Physiol,* 7, 361.

Rudwill, F., O'Gorman, D., Lefai, E., Chery, I., Zahariev, A., Normand, S., Pagano, A. F., Chopard, A., Damiot, A., Laurens, C., Hodson, L., Canet-Soulas, E., Heer, M., Meuthen, P. F.,

Buehlmeier, J., Baecker, N., Meiller, L., Gauquelin-Koch, G., Blanc, S., Simon, C. and Bergouignan, A. (2018) 'Metabolic Inflexibility Is an Early Marker of Bed-Rest-Induced Glucose Intolerance Even When Fat Mass Is Stable', *J Clin Endocrinol Metab,* 103(5), 1910-1920.

Rusnak, F. and Mertz, P. (2000) 'Calcineurin: form and function', *Physiol Rev,* 80(4), 1483-521.

Russ, D. W., Vandenborne, K. and Binder-Macleod, S. A. (2002) 'Factors in fatigue during intermittent electrical stimulation of human skeletal muscle', *Journal of Applied Physiology,* 93(2), 469-478.

Russell, J. T. (2011) 'Imaging calcium signals in vivo: a powerful tool in physiology and pharmacology', Br J Pharmacol, 163(8), 1605-25.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P. and Meyuhas, O. (2005) 'Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis', *Genes Dev,* 19(18), 2199-211.

Ryall, J. G. and Lynch, G. S. (2008) 'The potential and the pitfalls of β-adrenoceptor agonists for the management of skeletal muscle wasting', *Pharmacology & Therapeutics,* 120(3), 219-232.

Ryder, J. W., Kawano, Y., Galuska, D., Fahlman, R., Wallberg-Henriksson, H., Charron, M. J. and Zierath, J. R. (1999) 'Postexercise glucose uptake and glycogen synthesis in skeletal muscle from GLUT4-deficient mice', *The FASEB Journal,* 13(15), 2246-2256.

Ryder, J. W., Long, Y. C., Nilsson, E., Mahlapuu, M. and Zierath, J. R. (2005) 'Effects of calcineurin activation on insulin-, AICAR- and contraction-induced glucose transport in skeletal muscle', *J Physiol,* 567(Pt 2), 379-86.

Saini, J., Faroni, A., Reid, A. J., Mouly, V., Butler-Browne, G., Lightfoot, A. P., McPhee, J. S., Degens, H. and Al-Shanti, N. (2021) 'Cross-talk between motor neurons and myotubes via endogenously secreted neural and muscular growth factors', *Physiological Reports,* 9(8), e14791.

Saito, T., Okada, S., Shimoda, Y., Tagaya, Y., Osaki, A., Yamada, E., Shibusawa, R., Nakajima, Y., Ozawa, A., Satoh, T., Mori, M. and Yamada, M. (2016) 'APPL1 promotes glucose uptake in response to mechanical stretch via the PKCζ-non-muscle myosin IIa pathway in C2C12 myotubes', *Cellular Signalling,* 28(11), 1694-1702.

Saito, T., Okada, S., Shimoda, Y., Tagaya, Y., Osaki, A., Yamada, E., Shibusawa, R., Nakajima, Y., Ozawa, A., Satoh, T., Mori, M. and Yamada, M. (2016) 'APPL1 promotes glucose uptake in response to mechanical stretch via the PKCζ-non-muscle myosin IIa pathway in C2C12 myotubes', Cellular Signalling, 28(11), 1694-1702.

Sajan, M. P., Bandyopadhyay, G., Miura, A., Standaert, M. L., Nimal, S., Longnus, S. L., Van Obberghen, E., Hainault, I., Foufelle, F., Kahn, R., Braun, U., Leitges, M. and Farese, R. V. (2009) 'AICAR and metformin, but not exercise, increase muscle glucose transport through AMPK-, ERK-, and PDK1-dependent

activation of atypical PKC', *American Journal of Physiology-Endocrinology and Metabolism,* 298(2), E179-E192.

Sakamoto, K., Aschenbach, W. G., Hirshman, M. F. and Goodyear, L. J. (2003) 'Akt signalling in skeletal muscle: regulation by exercise and passive stretch', *American Journal of Physiology-Endocrinology and Metabolism,* 285(5), E1081-E1088.

Salanova, M., Volpe, P. and Blottner, D. (2013) 'Homer protein family regulation in skeletal muscle and neuromuscular adaptation', *IUBMB Life,* 65(9), 769-776.

Salvi, A. M., Bays, J. L., Mackin, S. R., Mege, R. M. and DeMali, K. A. (2021) 'Ankyrin G organizes membrane components to promote coupling of cell mechanics and glucose uptake', *Nat Cell Biol,* 23(5), 457-466.

Sanchez-Rangel, E. and Inzucchi, S. E. (2017) 'Metformin: clinical use in type 2 diabetes', *Diabetologia,* 60(9), 1586-1593.

Sandri, M. (2013) 'Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome', *Int J Biochem Cell Biol,* 45(10), 2121-9.

Sanvee, G. M., Bouitbir, J. and Krähenbühl, S. (2019) 'Insulin prevents and reverts simvastatin-induced toxicity in C2C12 skeletal muscle cells', *Sci Rep,* 9(1), 7409.

Sartori, R., Romanello, V. and Sandri, M. (2021) 'Mechanisms of muscle atrophy and hypertrophy: implications in health and disease', *Nat Commun,* 12(1), 330.

Sasaki, H., Kasagi, F., Yamada, M. and Fujita, S. (2007) 'Grip Strength Predicts Cause-Specific Mortality in Middle-Aged and Elderly Persons', *The American Journal of Medicine,* 120(4), 337-342.

Sase, K., Kido, K., Ato, S. and Fujita, S. (2019) 'The effect of a bout of resistance exercise on skeletal muscle protein metabolism after severe fasting', *Physiological Reports,* 7(21), e14270.

Scalabrin, M., Adams, V., Labeit, S. and Bowen, T. S. (2020) 'Emerging Strategies Targeting Catabolic Muscle Stress Relief', *Int J Mol Sci,* 21(13).

Scheler, M., Irmler, M., Lehr, S., Hartwig, S., Staiger, H., Al-Hasani, H., Beckers, J., Hrabé de Angelis, M., Häring, H.-U. and Weigert, C. (2013) 'Cytokine response of primary human myotubes in an in vitro exercise model', *American Journal of Physiology-Cell Physiology,* 305(8), C877-C886.

Schiaffino, S. and Mammucari, C. (2011) 'Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models', *Skelet Muscle,* 1(1), 4.

Schleh, M. W., Pitchford, L. M., Gillen, J. B. and Horowitz, J. F. (2020) 'Energy Deficit Required for Exercise-induced Improvements in Glycemia the Next Day', *Med Sci Sports Exerc,* 52(4), 976-982.

Schmidt-Arras, D. and Rose-John, S. (2016) 'IL-6 pathway in the liver: From physiopathology to therapy', *Journal of Hepatology,* 64(6), 1403-1415.

Schmiedeknecht, K., Kaufmann, A., Bauer, S. and Venegas Solis, F. (2022) 'L-lactate as an indicator for cellular metabolic status: An easy and cost-effective colorimetric L-lactate assay', *PLoS One,* 17(7), e0271818.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012) 'NIH Image to ImageJ: 25 years of image analysis', *Nature Methods,* 9(7), 671-675.

Scholpa, N. E., Simmons, E. C., Tilley, D. G. and Schnellmann, R. G. (2019) 'β2-adrenergic receptor-mediated mitochondrial biogenesis improves skeletal muscle recovery following spinal cord injury', *Experimental Neurology,* 322, 113064.

Schultze, S. M., Hemmings, B. A., Niessen, M. and Tschopp, O. C. e. (2012) 'PI3K/AKT, MAPK and AMPK signalling: protein kinases in glucose homeostasis', *Expert Reviews in Molecular Medicine,* 14, e1.

Schwander, M., Leu, M., Stumm, M., Dorchies, O. M., Ruegg, U. T., Schittny, J. and Müller, U. (2003) 'β1 Integrins Regulate Myoblast Fusion and Sarcomere Assembly', *Developmental Cell,* 4(5), 673-685.

Sedwick, C. (2019) 'Small calcium leaks, big muscle adaptations' in *J Gen Physiol*, United States: © 2019 Rockefeller University Press., 395.

Seedhouse, E. (1999) 'Running to Mars: Exercise countermeasures for Mars astronauts', *Proceedings of the Founding Convention of the Mars Society, Pt Iii,* 787-790.

Sekiguchi, F., Shimamura, K., Kawata, K., Nakazawa, Y., Saitoh, R., Yanagitani, Y. and Sunano, S. (1999) 'Effects of cyclopiazonic acid on contraction and intracellular Ca2+ in oesophageal striated muscle of normotensive and spontaneously hypertensive rats', Br J Pharmacol, 128(5), 961-8.

Severino, C., Brizzi, P., Solinas, A., Secchi, G., Maioli, M. and Tonolo, G. (2002) 'Low-dose dexamethasone in the rat: a model to study insulin resistance', American Journal of Physiology-Endocrinology and Metabolism, 283(2), E367-E373.

Shackelford, L. C., LeBlanc, A. D., Driscoll, T. B., Evans, H. J., Rianon, N. J., Smith, S. M., Spector, E., Feeback, D. L. and Lai, D. (2004) 'Resistance exercise as a countermeasure to disuse-induced bone loss', *J Appl Physiol (1985),* 97(1), 119-29.

Shahgholian, N., KarimiFard, O. and Shahidi, S. (2015) 'Effects of aerobic exercise on blood glucose in continuous ambulatory peritoneal dialysis patients', *Iran J Nurs Midwifery Res,* 20(2), 165-70.

Sharlo, K., Tyganov, S. A., Tomilovskaya, E., Popov, D. V., Saveko, A. A. and Shenkman, B. S. (2021) 'Effects of Various Muscle Disuse States and Countermeasures on Muscle Molecular Signalling', Int J Mol Sci, 23(1).

Shaw, R. J. (2009) 'LKB1 and AMP-activated protein kinase control of mTOR signalling and growth', *Acta Physiol (Oxf),* 196(1), 65-80.

Sheehan, P., Denieffe, S., Murphy, N. M. and Harrison, M. (2019) 'Exercise is more effective than health education in reducing fatigue in fatigued cancer survivors', *Supportive Care in Cancer*, 10.

Shi, D., Gu, R., Song, Y., Ding, M., Huang, T., Guo, M., Xiao, J., Huang, W. and Liao, H. (2018) 'Calcium/Calmodulin-Dependent Protein Kinase IV (CaMKIV) Mediates Acute Skeletal Muscle Inflammatory Response', *Inflammation,* 41(1), 199-212.

Shur, N. F., Creedon, L., Skirrow, S., Atherton, P. J., MacDonald, I. A., Lund, J. and Greenhaff, P. L. 'Age-related changes in muscle architecture and metabolism in humans: The likely contribution of physical inactivity to age-related functional decline', (1872-9649 (Electronic)).

Shur, N. F., Simpson, E. J., Crossland, H., Chivaka, P. K., Constantin, D., Cordon, S. M., Constantin-Teodosiu, D., Stephens, F. B., Lobo, D. N., Szewczyk, N., Narici, M., Prats, C., Macdonald, I. A. and Greenhaff, P. L. (2022) 'Human adaptation to immobilization: Novel insights of impacts on glucose disposal and fuel utilization', J Cachexia Sarcopenia Muscle, 13(6), 2999-3013.

Sibonga, J. D. (2013) 'Spaceflight-induced Bone Loss: Is there an Osteoporosis Risk?', *Current Osteoporosis Reports,* 11(2), 92-98.

Sidorenko, S., Klimanova, E., Milovanova, K., Lopina, O. D., Kapilevich, L. V., Chibalin, A. V. and Orlov, S. N. (2018) 'Transcriptomic changes in C2C12 myotubes triggered by electrical stimulation: Role of Ca2+i-mediated and Ca2+i-independent signalling and elevated [Na+]i/[K+]i ratio', *Cell Calcium,* 76, 72-86.

Singh, R., Gupta, V., Kumar, A. and Singh, K. (2023) '2-Deoxy-D-Glucose: A Novel Pharmacological Agent for Killing Hypoxic Tumor Cells, Oxygen Dependence-Lowering in Covid-19, and Other Pharmacological Activities', *Advances in Pharmacological and Pharmaceutical Sciences,* 2023, 9993386.

Sivitz, W. I. and Yorek, M. A. (2010) 'Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities', *Antioxid Redox Signal,* 12(4), 537-77.

Smith, S. M., Zwart, S. R., Block, G., Rice, B. L. and Davis-Street, J. E. (2005) 'The nutritional status of astronauts is altered after long-term space flight aboard the International Space Station', *J Nutr,* 135(3), 437-43.

Smith, S. M., Zwart, S. R., Block, G., Rice, B. L. and Davis-Street, J. E. (2005) 'The nutritional status of astronauts is altered after long-term space flight aboard the International Space Station', *J Nutr,* 135(3), 437-43.

Snijders, T., Nederveen, J. P., McKay, B. R., Joanisse, S., Verdijk, L. B., van Loon, L. J. and Parise, G. (2015) 'Satellite cells in human skeletal muscle plasticity', *Front Physiol,* 6, 283.

Son, Y. H., Lee, S.-M., Lee, S. H., Yoon, J. H., Kang, J. S., Yang, Y. R. and Kwon, K.-S. (2019) 'Comparative molecular analysis of endurance exercise in vivo with electrically stimulated in vitro myotube contraction', *Journal of Applied Physiology,* 127(6), 1742-1753.

Soo, J., Raman, A., Lawler, N. G., Goods, P. S. R., Deldicque, L., Girard, O. and Fairchild, T. J. (2023a) 'The role of exercise and hypoxia on glucose transport and regulation', *European Journal of Applied Physiology,* 123(6), 1147-1165.

Soo, J., Raman, A., Lawler, N. G., Goods, P. S. R., Deldicque, L., Girard, O. and Fairchild, T. J. (2023b) 'The role of exercise and hypoxia on glucose transport and regulation', *European Journal of Applied Physiology,* 123(6), 1147-1165.

Sorriento, D., Di Vaia, E. and Iaccarino, G. (2021) 'Physical Exercise: A Novel Tool to Protect Mitochondrial Health', *Front Physiol,* 12, 660068.

Speacht, T. L., Krause, A. R., Steiner, J. L., Lang, C. H. and Donahue, H. J. (2018) 'Combination of hindlimb suspension and immobilization by casting exaggerates sarcopenia by stimulating autophagy but does not worsen osteopenia', Bone, 110, 29-37.

Stallknecht, B., Vissing, J. and Galbo, H. (1998) 'Lactate production and clearance in exercise. Effects of training. A mini-review', *Scand J Med Sci Sports,* 8(3), 127-31.

Stallknecht, B., Vissing, J. and Galbo, H. (1998a) 'Lactate production and clearance in exercise. Effects of training. A mini-review', *Scandinavian Journal of Medicine & Science in Sports,* 8(3), 127-131.

Stallknecht, B., Vissing, J. and Galbo, H. (1998b) 'Lactate production and clearance in exercise. Effects of training. A mini-review', *Scandinavian Journal of Medicine & Science in Sports,* 8(3), 127-131.

Stanford, K. I. and Goodyear, L. J. (2018) 'Muscle-Adipose Tissue Cross Talk', *Cold Spring Harb Perspect Med,* 8(8).

Steele, J., Androulakis-Korakakis, P., Perrin, C., Fisher, J. P., Gentil, P., Scott, C. and Rosenberger, A. (2019) 'Comparisons of Resistance Training and "Cardio" Exercise Modalities as Countermeasures to Microgravity-Induced Physical Deconditioning: New Perspectives and Lessons Learned From Terrestrial Studies', *Frontiers in Physiology,* 10, 12.

Steensberg, A., Febbraio, M. A., Osada, T., Schjerling, P., van Hall, G., Saltin, B. and Pedersen, B. K. (2001) 'Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content', *J Physiol,* 537(Pt 2), 633-9.

Stein, T. P. (2013) 'Weight, muscle and bone loss during space flight: another perspective', *Eur J Appl Physiol,* 113(9), 2171-81.

Strollo, F., Gentile, S., Strollo, G., Mambro, A. and Vernikos, J. (2018) 'Recent Progress in Space Physiology and Aging', *Front Physiol,* 9, 1551.

Stuart, C. A., Shangraw, R. E., Prince, M. J., Peters, E. J. and Wolfe, R. R. (1988) 'Bed-rest-induced insulin resistance occurs primarily in muscle', *Metabolism,* 37(8), 802-6.

Stuart, C. A., Shangraw, R. E., Prince, M. J., Peters, E. J. and Wolfe, R. R. (1988) 'Bed-rest-induced insulin resistance occurs primarily in muscle', Metabolism, 37(8), 802-6.

Suginohara, T., Wakabayashi, K., Ato, S. and Ogasawara, R. (2021) 'Effect of 2-deoxyglucose-mediated inhibition of glycolysis on the regulation of mTOR signalling and protein synthesis before and after high-intensity muscle contraction', *Metabolism,* 114, 154419.

Suh, J. and Lee, Y. S. (2020) 'Myostatin Inhibitors: Panacea or Predicament for Musculoskeletal Disorders?', *J Bone Metab,* 27(3), 151-165.

Sylow, L., Kleinert, M., Richter, E. A. and Jensen, T. E. (2017) 'Exercise-stimulated glucose uptake — regulation and implications for glycaemic control', *Nature Reviews Endocrinology,* 13(3), 133-148.

Tabozzi, S. A., Di Santo, S. G., Franchini, F., Ratto, F., Luchi, M., Filiputti, B., Ardigò, L. P. and Lafortuna, C. L. (2020) 'Sedentary Behaviors and Physical Activity Habits Independently Affect Fat Oxidation in Fasting Conditions and Capillary Glucose Levels After Standardized Glucose-Rich Meal in Healthy Females', *Frontiers in Physiology,* 11.

Tahapary, D. L., Pratisthita, L. B., Fitri, N. A., Marcella, C., Wafa, S., Kurniawan, F., Rizka, A., Tarigan, T. J. E., Harbuwono, D. S., Purnamasari, D. and Soewondo,

P. (2022) 'Challenges in the diagnosis of insulin resistance: Focusing on the role of HOMA-IR and Tryglyceride/glucose index', *Diabetes & Metabolic Syndrome: Clinical Research & Reviews,* 16(8), 102581.

Takimoto, M., Takeyama, M. and Hamada, T. (2013) 'Possible involvement of AMPK in acute exercise-induced expression of monocarboxylate transporters MCT1 and MCT4 mRNA in fast-twitch skeletal muscle', *Metabolism,* 62(11), 1633-1640.

Tammineni, E. R., Kraeva, N., Figueroa, L., Manno, C., Ibarra, C. A., Klip, A., Riazi, S. and Rios, E. (2020) 'Intracellular calcium leak lowers glucose storage in human muscle, promoting hyperglycemia and diabetes', *Elife,* 9.

Tammineni, E. R., Kraeva, N., Figueroa, L., Manno, C., Ibarra, C. A., Klip, A., Riazi, S. and Rios, E. (2020a) 'Intracellular calcium leak lowers glucose storage in human muscle, promoting hyperglycemia and diabetes', Elife, 9.

Tammineni, E. R., Kraeva, N., Figueroa, L., Manno, C., Ibarra, C. A., Klip, A., Riazi, S. and Rios, E. (2020b) 'Intracellular calcium leak lowers glucose storage in human muscle, promoting hyperglycemia and diabetes', Elife, 9.

Tamura, Y., Kouzaki, K., Kotani, T. and Nakazato, K. (2020) 'Electrically stimulated contractile activity-induced transcriptomic responses and metabolic remodeling in C2C12 myotubes: twitch vs. tetanic contractions', *American Journal of Physiology-Cell Physiology,* 319(6), C1029-C1044.

Tarpey, M. D., Amorese, A. J., Balestrieri, N. P., Fisher-Wellman, K. H. and Spangenburg, E. E. (2019) 'Doxorubicin causes lesions in the electron transport system of skeletal muscle mitochondria that are associated with a loss of contractile function', *J Biol Chem,* 294(51), 19709-19722.

Tavi, P. and Westerblad, H. (2011) 'The role of in vivo Ca^{2+} signals acting on Ca^{2+}-calmodulin-dependent proteins for skeletal muscle plasticity', *J Physiol,* 589(Pt 21), 5021-31.

Taylor, L. W., Wilborn, C. D., Kreider, R. B. and Willoughby, D. S. (2012) 'Effects of resistance exercise intensity on extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase activation in men', *J Strength Cond Res,* 26(3), 599-607.

Taylor, R. (2012) 'Insulin resistance and type 2 diabetes', *Diabetes,* 61(4), 778-9.

Taylor, S. R., Lopez, J. R., Griffiths, P. J., Trube, G. and Cecchi, G. (1982) 'Calcium in excitation--contraction coupling of frog skeletal muscle', *Can J Physiol Pharmacol,* 60(4), 489-502.

Teodori, L., Costa, A., Campanella, L. and Albertini, M. C. (2018) 'Skeletal Muscle Atrophy in Simulated Microgravity Might Be Triggered by Immune-Related microRNAs', *Front Physiol,* 9, 1926.

TeSlaa, T. and Teitell, M. A. (2014) 'Techniques to monitor glycolysis', *Methods Enzymol,* 542, 91-114.

Tesseraux, I., Gulden, M. and Wassermann, O. (1987) 'Cultured myotubes from skeletal muscle of adult rats. Characterization and action of Anemonia sulcata toxin II', *Naunyn Schmiedebergs Arch Pharmacol,* 336(2), 232-9.

Thirsk, R., Kuipers, A., Mukai, C. and Williams, D. (2009) 'The space-flight environment: the International Space Station and beyond', *Cmaj,* 180(12), 1216-20.

Thirupathi, A. and Pinho, R. A. (2018) 'Effects of reactive oxygen species and interplay of antioxidants during physical exercise in skeletal muscles', *J Physiol Biochem,* 74(3), 359-367.

Thom, J. M., Thompson, M. W., Ruell, P. A., Bryant, G. J., Fonda, J. S., Harmer, A. R., De Jonge, X. A. K. J. and Hunter, S. K. (2001a) 'Effect of 10-day cast immobilization on sarcoplasmic reticulum calcium regulation in humans', *Acta Physiologica Scandinavica,* 172(2), 141-147.

Thom, J. M., Thompson, M. W., Ruell, P. A., Bryant, G. J., Fonda, J. S., Harmer, A. R., Janse de Jonge, X. A. and Hunter, S. K. (2001b) 'Effect of 10-day cast immobilization on sarcoplasmic reticulum calcium regulation in humans', *Acta Physiol Scand,* 172(2), 141-7.

Thomson, D. M. (2018) 'The Role of AMPK in the Regulation of Skeletal Muscle Size, Hypertrophy, and Regeneration', *International Journal of Molecular Sciences,* 19(10), 20.

Tjonna, A. E., Lee, S. J., Rognmo, O., Stolen, T. O., Bye, A., Haram, P. M., Loennechen, J. P., Al-Share, Q. Y., Skogvoll, E., Slordahl, S. A., Kemi, O. J., Najjar, S. M. and Wisloff, U. (2008) 'Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome - A pilot study', *Circulation,* 118(4), 346-354.

Tobin, B. W., Uchakin, P. N. and Leeper-Woodford, S. K. (2002) 'Insulin secretion and sensitivity in space flight: diabetogenic effects', *Nutrition,* 18(10), 842-8.

Tofas, T., Draganidis, D., Deli, C. K., Georgakouli, K., Fatouros, I. G. and Jamurtas, A. Z. (2019) 'Exercise-Induced Regulation of Redox Status in Cardiovascular Diseases: The Role of Exercise Training and Detraining', *Antioxidants (Basel),* 9(1).

Tokuda, K., Yamanaka, Y., Mano, Y., Tsukamoto, M., Tajima, T., Suzuki, H., Kawasaki, M., Uchida, S., Nakamura, E., Wang, K. Y. and Sakai, A. (2021) 'Effect of metformin treatment and its time of administration on joint capsular fibrosis induced by mouse knee immobilization', *Sci Rep,* 11(1), 17978.

Tomilovskaya, E., Shigueva, T., Sayenko, D., Rukavishnikov, I. and Kozlovskaya, I. (2019) 'Dry Immersion as a Ground-Based Model of Microgravity Physiological Effects', *Frontiers in Physiology,* 10.

Tortorella, L. L. and Pilch, P. F. (2002) 'C2C12 myocytes lack an insulin-responsive vesicular compartment despite dexamethasone-induced GLUT4 expression', *Am J Physiol Endocrinol Metab,* 283(3), E514-24.

Trappe, T., Trappe, S., Lee, G., Widrick, J., Fitts, R. and Costill, D. (2006) 'Cardiorespiratory responses to physical work during and following 17 days of bed rest and spaceflight', *J Appl Physiol (1985),* 100(3), 951-7.

Trefts, E., Williams, A. S. and Wasserman, D. H. (2015) 'Exercise and the Regulation of Hepatic Metabolism', *Prog Mol Biol Transl Sci,* 135, 203-25.

Triplitt, C. L. (2012) 'Examining the mechanisms of glucose regulation', Am J Manag Care, 18(1 Suppl), S4-10.

Tsai, S., Sitzmann, J. M., Dastidar, S. G., Rodriguez, A. A., Vu, S. L., McDonald, C. E., Academia, E. C., O'Leary, M. N., Ashe, T. D., La Spada, A. R. and Kennedy, B. K. (2015) 'Muscle-specific 4E-BP1 signalling activation improves metabolic parameters during aging and obesity', *J Clin Invest,* 125(8), 2952-64.

Tsai, S., Sitzmann, J. M., Dastidar, S. G., Rodriguez, A. A., Vu, S. L., McDonald, C. E., Academia, E. C., O'Leary, M. N., Ashe, T. D., La Spada, A. R. and Kennedy, B. K. (2015) 'Muscle-specific 4E-BP1 signalling activation improves metabolic parameters during aging and obesity', J Clin Invest, 125(8), 2952-64.

Tsukamoto, S., Shibasaki, A., Naka, A., Saito, H. and Iida, K. (2018) 'Lactate Promotes Myoblast Differentiation and Myotube Hypertrophy via a Pathway Involving MyoD In Vitro and Enhances Muscle Regeneration In Vivo', *Int J Mol Sci,* 19(11).

Tsutaki, A., Ogasawara, R., Kobayashi, K., Lee, K., Kouzaki, K. and Nakazato, K. (2013) 'Effect of Intermittent Low-Frequency Electrical Stimulation on the Rat Gastrocnemius Muscle', *BioMed Research International,* 2013, 480620.

Tu, M. K., Levin, J. B., Hamilton, A. M. and Borodinsky, L. N. (2016) 'Calcium signaling in skeletal muscle development, maintenance and regeneration', *Cell Calcium,* 59(2-3), 91-7.

Turinsky, J., Nagel, G. W., Elmendorf, J. S., Damrau-Abney, A. and Smith, T. R. (1996) 'Sphingomyelinase stimulates 2-deoxyglucose uptake by skeletal muscle', *Biochem J,* 313 (Pt 1)(Pt 1), 215-22.

Uchimura, T., Asano, T., Nakata, T., Hotta, A. and Sakurai, H. (2021) 'A muscle fatigue-like contractile decline was recapitulated using skeletal myotubes from Duchenne muscular dystrophy patient-derived iPSCs', *Cell Reports Medicine,* 2(6).

UK Chief Medical Officers' Physical Activity Guidelines. Available online at [https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/832868/uk-chief-medical-officers-physical-activity-guidelines.pdf]

Uryash, A., Mijares, A., Lopez, C. E., Adams, J. A. and Lopez, J. R. (2022) 'Chronic Elevation of Skeletal Muscle [Ca2+]i Impairs Glucose Uptake. An in Vivo and in Vitro Study', Frontiers in Physiology, 13.

Vainshtein, A., Tryon, L. D., Pauly, M. and Hood, D. A. (2015) 'Role of PGC-1α during acute exercise-induced autophagy and mitophagy in skeletal muscle', Am J Physiol Cell Physiol, 308(9), C710-9.

Valenta, T., Hausmann, G. and Basler, K. (2012) 'The many faces and functions of β-catenin', *Embo j,* 31(12), 2714-36.

van Dijk, J. W. and van Loon, L. J. (2015) 'Exercise strategies to optimize glycemic control in type 2 diabetes: a continuing glucose monitoring perspective', *Diabetes Spectr,* 28(1), 24-31.

Venojärvi, M., Korkmaz, A., Aunola, S., Hällsten, K., Virtanen, K., Marniemi, J., Halonen, J. P., Hänninen, O., Nuutila, P. and Atalay, M. (2014) 'Decreased thioredoxin-1 and increased HSP90 expression in skeletal muscle in subjects with type 2 diabetes or impaired glucose tolerance', *Biomed Res Int,* 2014, 386351.

Verdijk, L. B., Snijders, T., Drost, M., Delhaas, T., Kadi, F. and van Loon, L. J. (2014) 'Satellite cells in human skeletal muscle; from birth to old age', *Age (Dordr),* 36(2), 545-7.

Vilchinskaya, N. A., Mirzoev, T. M., Lomonosova, Y. N., Kozlovskaya, I. B. and Shenkman, B. S. (2015) 'Human muscle signalling responses to 3-day head-out dry immersion', J Musculoskelet Neuronal Interact, 15(3), 286-93.

Villella, M. and Villella, A. (2014) 'Exercise and cardiovascular diseases', *Kidney Blood Press Res,* 39(2-3), 147-53.

Vissing, J., Ohkuwa, T., Ploug, T. and Galbo, H. (1988) 'Effect of prior immobilization on muscular glucose clearance in resting and running rats', *American Journal of Physiology-Endocrinology and Metabolism,* 255(4), E456-E462.

Vissing, K., McGee, S., Farup, J., Kjølhede, T., Vendelbo, M. and Jessen, N. (2013) 'Differentiated mTOR but not AMPK signalling after strength vs endurance exercise in training-accustomed individuals', *Scand J Med Sci Sports,* 23(3), 355-66.

Vitzel, K. F., Bikopoulos, G., Hung, S., Pistor, K. E., Patterson, J. D., Curi, R. and Ceddia, R. B. (2013) 'Chronic treatment with the AMP-kinase activator AICAR

increases glycogen storage and fatty acid oxidation in skeletal muscles but does not reduce hyperglucagonemia and hyperglycemia in insulin deficient rats', *PLoS One,* 8(4), e62190.

Vlassara, H. and Uribarri, J. (2014) 'Advanced glycation end products (AGE) and diabetes: cause, effect, or both?', *Curr Diab Rep,* 14(1), 453.

WAGENMAKERS, A. J. M. (1998) '11 Muscle Amino Acid Metabolism at Rest and During Exercise: Role in Human Physiology and Metabolism', *Exercise and Sport Sciences Reviews,* 26(1), 287-314.

Wahl, M. P., Scalzo, R. L., Regensteiner, J. G. and Reusch, J. E. B. (2018) 'Mechanisms of Aerobic Exercise Impairment in Diabetes: A Narrative Review', *Frontiers in Endocrinology,* 9.

Wakizaka, M., Eshima, H., Tanaka, Y., Shirakawa, H., Poole, D. C. and Kano, Y. (2017) 'In vivo Ca2+ dynamics induced by Ca2+ injection in individual rat skeletal muscle fibers', Physiological Reports, 5(5), e13180.

Wall, B. T., Dirks, M. L., Snijders, T., Senden, J. M. G., Dolmans, J. and van Loon, L. J. C. (2014) 'Substantial skeletal muscle loss occurs during only 5 days of disuse', *Acta Physiologica,* 210(3), 600-611.

Walton, R. G., Dungan, C. M., Long, D. E., Tuggle, S. C., Kosmac, K., Peck, B. D., Bush, H. M., Villasante Tezanos, A. G., McGwin, G., Windham, S. T., Ovalle, F., Bamman, M. M., Kern, P. A. and Peterson, C. A. (2019) 'Metformin blunts muscle hypertrophy in response to progressive resistance exercise training in older adults: A randomized, double-blind, placebo-controlled, multicenter trial: The MASTERS trial', *Aging Cell,* 18(6), e13039.

Wang, B. Y., Hsiao, A. W., Wong, N., Chen, Y. F., Lee, C. W. and Lee, W. Y. W. (2023) 'Is dexamethasone-induced muscle atrophy an alternative model for naturally aged sarcopenia model?', *J Orthop Translat,* 39, 12-20.

Wang, C., Liu, F., Yuan, Y., Wu, J., Wang, H., Zhang, L., Hu, P., Li, Z., Li, Q. and Ye, J. (2014) 'Metformin suppresses lipid accumulation in skeletal muscle by promoting fatty acid oxidation', *Clin Lab,* 60(6), 887-96.

Wang, F., Zhang, Q. B., Zhou, Y., Chen, S., Huang, P. P., Liu, Y. and Xu, Y. H. (2019) 'The mechanisms and treatments of muscular pathological changes in immobilization-induced joint contracture: A literature review', *Chin J Traumatol,* 22(2), 93-98.

Wang, L., Wang, Z., Yang, K., Shu, G., Wang, S., Gao, P., Zhu, X., Xi, Q., Zhang, Y. and Jiang, Q. (2016) 'Epigallocatechin Gallate Reduces Slow-Twitch Muscle Fiber Formation and Mitochondrial Biosynthesis in C2C12 Cells by Repressing AMPK Activity and PGC-1alpha Expression', *J Agric Food Chem,* 64(34), 6517-23.

Wang, X., Hu, Z., Hu, J., Du, J. and Mitch, W. E. (2006) 'Insulin Resistance Accelerates Muscle Protein Degradation: Activation of the Ubiquitin-Proteasome Pathway by Defects in Muscle Cell Signaling', *Endocrinology,* 147(9), 4160-4168.

Wang, Y. W., He, S. J., Feng, X., Cheng, J., Luo, Y. T., Tian, L. and Huang, Q. (2017) 'Metformin: a review of its potential indications', *Drug Des Devel Ther,* 11, 2421-2429.

Wannenes, F., Magni, L., Bonini, M., Dimauro, I., Caporossi, D., Moretti, C. and Bonini, S. (2012) 'In Vitro Effects of Beta-2 Agonists on Skeletal Muscle Differentiation, Hypertrophy, and Atrophy', *World Allergy Organization Journal,* 5(6), 66-72.

Wasserman, D. H. (2021) 'Insulin, Muscle Glucose Uptake, and Hexokinase: Revisiting the Road Not Taken', *Physiology,* 37(3), 115-127.

Watford, M. (2015) 'Glutamine and glutamate: Nonessential or essential amino acids?', *Anim Nutr,* 1(3), 119-122.

Watson, K. and Baar, K. (2014) 'mTOR and the health benefits of exercise', *Seminars in Cell & Developmental Biology,* 36, 130-139.

Weber, T., Green, D. A., Attias, J., Sies, W., Frechette, A., Braunstein, B. and Rittweger, J. (2019) 'Hopping in hypogravity-A rationale for a plyometric exercise countermeasure in planetary exploration missions', *PLoS One,* 14(2), e0211263.

Weigert, C., Hennige, A. M., Brodbeck, K., Häring, H. U. and Schleicher, E. D. (2005) 'Interleukin-6 acts as insulin sensitizer on glycogen synthesis in human skeletal muscle cells by phosphorylation of Ser473 of Akt', *American Journal of Physiology-Endocrinology and Metabolism,* 289(2), E251-E257.

Welsh, P., Rankin, N., Li, Q., Mark, P. B., Wurtz, P., Ala-Korpela, M., Marre, M., Poulter, N., Hamet, P., Chalmers, J., Woodward, M. and Sattar, N. (2018) 'Circulating amino acids and the risk of macrovascular, microvascular and mortality outcomes in individuals with type 2 diabetes: results from the ADVANCE trial', *Diabetologia,* 61(7), 1581-1591.

Williams, G. S. B., Boyman, L., Chikando, A. C., Khairallah, R. J. and Lederer, W. J. (2013) 'Mitochondrial calcium uptake', *Proceedings of the National Academy of Sciences,* 110(26), 10479-10486.

Winslow, M. A. and Hall, S. E. (2019) 'Muscle wasting: A review of exercise, classical and non-classical RAS axes', *Journal of Cellular and Molecular Medicine,* 23(9), 5836-5845.

Winslow, M. A. and Hall, S. E. (2019) 'Muscle wasting: A review of exercise, classical and non-classical RAS axes', *Journal of Cellular and Molecular Medicine,* 23(9), 5836-5845.

Witczak, C. A., Jessen, N., Warro, D. M., Toyoda, T., Fujii, N., Anderson, M. E., Hirshman, M. F. and Goodyear, L. J. (2010) 'CaMKII regulates contraction- but not insulin-induced glucose uptake in mouse skeletal muscle', American Journal of Physiology-Endocrinology and Metabolism, 298(6), E1150-E1160.

Wollheim, C. B. and Sharp, G. W. G. (1981) 'REGULATION OF INSULIN RELEASE BY CALCIUM', *Physiological Reviews,* 61(4), 914-973.

Wong, C. Y., Al-Salami, H. and Dass, C. R. (2020) 'C2C12 cell model: its role in understanding of insulin resistance at the molecular level and pharmaceutical development at the preclinical stage', *Journal of Pharmacy and Pharmacology,* 72(12), 1667-1693.

Wotring, V. E. (2015) 'Medication use by U.S. crewmembers on the International Space Station', *The FASEB Journal,* 29(11), 4417-4423.

Wu, M., Falasca, M. and Blough, E. R. (2011) 'Akt/protein kinase B in skeletal muscle physiology and pathology', *Journal of Cellular Physiology,* 226(1), 29-36.

Wuest, S. L., Richard, S., Kopp, S., Grimm, D. and Egli, M. (2015) 'Simulated microgravity: critical review on the use of random positioning machines for mammalian cell culture', *Biomed Res Int,* 2015, 971474.

Xi, H., Kurtoglu, M. and Lampidis, T. J. (2014) 'The wonders of 2-deoxy-d-glucose', *IUBMB Life,* 66(2), 110-121.

Xu, L., Tripathy, A., Pasek, D. A. and Meissner, G. (1999) 'Ruthenium Red Modifies the Cardiac and Skeletal Muscle Ca2+ Release Channels (Ryanodine Receptors) by Multiple Mechanisms', *Journal of Biological Chemistry,* 274(46), 32680-32691.

Yang, D. Q. and Kastan, M. B. (2000) 'Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1', *Nat Cell Biol,* 2(12), 893-8.

Yang, L., Tautz, T., Zhang, S., Fomina, A. and Liu, H. (2019) 'The current status of malignant hyperthermia', *J Biomed Res,* 34(2), 75-85.

Yoon, M. S. (2016) 'The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism', *Nutrients,* 8(7).

You, J. S., Anderson, G. B., Dooley, M. S. and Hornberger, T. A. (2015) 'The role of mTOR signalling in the regulation of protein synthesis and muscle mass during immobilization in mice', Dis Model Mech, 8(9), 1059-69.

Youn, J. H., Gulve, E. A. and Holloszy, J. O. (1991a) 'Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction', American Journal of Physiology-Cell Physiology, 260(3), C555-C561.

Youn, J. H., Gulve, E. A. and Holloszy, J. O. (1991b) 'Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction', American Journal of Physiology-Cell Physiology, 260(3), C555-C561.

Yu, M., Blomstrand, E., Chibalin, A. V., Krook, A. and Zierath, J. R. (2001) 'Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle', *J Physiol,* 536(Pt 1), 273-82.

Yun, H., Lee, J. H., Park, C. E., Kim, M. J., Min, B. I., Bae, H., Choe, W., Kang, I., Kim, S. S. and Ha, J. (2009) 'Inulin increases glucose transport in C2C12 myotubes and HepG2 cells via activation of AMP-activated protein kinase and phosphatidylinositol 3-kinase pathways', *J Med Food,* 12(5), 1023-8.

Yun, Y. S., Kim, K. H., Tschida, B., Sachs, Z., Noble-Orcutt, K. E., Moriarity, B. S., Ai, T., Ding, R., Williams, J., Chen, L., Largaespada, D. and Kim, D. H. (2016) 'mTORC1 Coordinates Protein Synthesis and Immunoproteasome Formation via PRAS40 to Prevent Accumulation of Protein Stress', *Mol Cell,* 61(4), 625-639.

Zaid, H., Antonescu, C., Randhawa, V. and Klip, A. (2008a) 'Insulin action on glucose transporters through molecular switches, tracks and tethers', *The Biochemical journal,* 413, 201-15.

Zaid, H., Antonescu, Costin N., Randhawa, Varinder K. and Klip, A. (2008b) 'Insulin action on glucose transporters through molecular switches, tracks and tethers', *Biochemical Journal,* 413(2), 201-215.

Zanou, N., Dridi, H., Reiken, S., Imamura de Lima, T., Donnelly, C., De Marchi, U., Ferrini, M., Vidal, J., Sittenfeld, L., Feige, J. N., Garcia-Roves, P. M., Lopez-Mejia, I. C., Marks, A. R., Auwerx, J., Kayser, B. and Place, N. (2021) 'Acute RyR1 Ca2+ leak enhances NADH-linked mitochondrial respiratory capacity', *Nature Communications,* 12(1), 7219.

Zbinden-Foncea, H., Raymackers, J. M., Deldicque, L., Renard, P. and Francaux, M. (2012) 'TLR2 and TLR4 activate p38 MAPK and JNK during endurance exercise in skeletal muscle', *Med Sci Sports Exerc,* 44(8), 1463-72.

Zebedin, E., Mille, M., Speiser, M., Zarrabi, T., Sandtner, W., Latzenhofer, B., Todt, H. and Hilber, K. (2007) 'C2C12 skeletal muscle cells adopt cardiac-like sodium current properties in a cardiac cell environment', *American Journal of Physiology-Heart and Circulatory Physiology,* 292(1), H439-H450.

Zhang, D., Li, J., Wang, F., Hu, J., Wang, S. and Sun, Y. (2014) '2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy', *Cancer Letters,* 355(2), 176-183.

Zhang, L., Wang, X. H., Wang, H., Du, J. and Mitch, W. E. (2010) 'Satellite cell dysfunction and impaired IGF-1 signalling cause CKD-induced muscle atrophy', *J Am Soc Nephrol,* 21(3), 419-27.

Zhang, R., Shen, Q., Wang, Y., Deng, X., Fan, J., Gu, X., Fan, M., Wei, K., Cheng, C. R., Zhang, W. D., Zhang, X. W. and Liu, X. (2023) 'Corylifol A ameliorates muscle atrophy by inhibiting TAOK1/p38-MAPK/FoxO3 pathway in cancer cachexia', *J Cachexia Sarcopenia Muscle*.

Zhao, C., Yu, Y., Zhang, Y., Shen, J., Jiang, L., Sheng, G., Zhang, W., Xu, L., Jiang, K., Mao, S., Jiang, P. and Gao, F. (2019) 'β-Catenin Controls the Electrophysiologic Properties of Skeletal Muscle Cells by Regulating the α2 Isoform of Na+/K+-ATPase', *Frontiers in Neuroscience,* 13.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J. and Moller, D. E. (2001) 'Role of AMP-activated protein kinase in mechanism of metformin action', *J Clin Invest,* 108(8), 1167-74.

Zhou, Y., Liu, X., Huang, C. and Lin, D. (2022) 'Lactate Activates AMPK Remodeling of the Cellular Metabolic Profile and Promotes the Proliferation and Differentiation of C2C12 Myoblasts', *International Journal of Molecular Sciences,* 23(22), 13996.

Zierath, J. R., He, L., Gumà, A., Odegoard Wahlström, E., Klip, A. and Wallberg-Henriksson, H. (1996) 'Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM', *Diabetologia,* 39(10), 1180-9.