Glutathione Depletion Affects the Expression of Genes Involved in the Molecular Adaptation of C2C12 Myotubes to Contraction

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of

Master of Science
in
Physiology

at Massey University, Auckland,
New Zealand.

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2021

Abstract

Background

Impaired muscle metabolic function and atrophy are closely linked to a wide range of conditions including cancer, diabetes, and aging. Exercise is known to improve muscle metabolism and increase muscle mass, thereby having protective and therapeutic effects against such conditions. During exercise the production of reactive oxygen species (ROS) within muscle increases. These highly reactive molecules are typically balanced by antioxidants and act as signalling molecules bringing about changes in gene expression. However, if the balance between production and detoxification is lost, they can cause oxidative damage. Chronic oxidative stress can cause muscle to atrophy and impair energy metabolism, contributing to the complications of the aforementioned conditions. Glutathione (GSH) is a three amino acid peptide and one of the most abundant antioxidant molecules in muscle, protecting against oxidative damage. In addition, when oxidised by ROS, GSH may interact with and change the activity of a range of other enzymes, affecting the activation of several signalling pathways that influence the expression of genes associated with energy metabolism and antioxidant capacity. This suggests that GSH may play a role in the magnitude of muscle adaptations to exercise through redox sensitive pathways.

Aim

The primary aim was to evaluate the involvement of endogenous GSH in the molecular adaptation of skeletal muscle to contractions induced by electrical stimulation (ES) *in vitro*.

Methods

C2C12 myoblasts were cultured and differentiated into myotubes. The experimental groups consisted of controls and those depleted of GSH by a 24-hour treatment with 1 mM buthionine sulphoximine (BSO). These were then submitted to an electrical stimulation protocol of a 1 second pulse every other second of 10 V and 50 Hz for 24 hours. Cell survival was tested using exclusion technique and trypan blue dye to determine if ES or treatment with BSO effected cell viability. Hydrogen peroxide release during ES was measured using a fluorescent probe (Amplex UltraRed©) and GSH content in the myotubes was quantified using a commercial kit. RT-PCR was conducted to measure the expression of key genes associated with muscle adaptation and metabolism

following contraction. The genes tested were Catalase, CPT-1B, Citrate Synthase, GLUT4, GPx1, HK2, NRF1. NRF2, PFK, PGC-1α, PPARα, PPARγ, SOD1, SOD2, Tfam, and VEGF-A. Baseline expression was compared to expression immediately following ES as well as 1 and 3 hours after stimulation had ended.

Results

The ES protocol nor treatment with BSO, alone or combined, caused any significant change in cell viability when compared to controls. Cellular concentrations of total GSH were successfully depleted in C2C12 myotubes treated with BSO. GSH depletion caused an increase in H_2O_2 concentrations of treated cells, both at baseline and during ES, in comparison to control groups. GSH depletion increased the expression of PPARγ across all time points when compared to control cells and tended to increase expression of multiple genes at baseline when compared to control groups (PFK $p = 0.1149$, PPARα $p = 0.0654$, and SOD1 $p = 0.086$; unpaired t-test). Electrical stimulation upregulated the expression of VEGF, GLUT4, and PGC-1α irrespective of GSH treatment, along with a tendency to increase the expression of CPT-1B ($p = 0.0580$; two-way ANOVA). Catalase mRNA levels increased over time following ES and were further increased in GSH depleted groups.

Discussion

GSH (or the absence of) may affect baseline expression of several genes known to be involved in the molecular adaptation of skeletal muscle to exercise but, following ES, GSH depletion only affected catalase expression. The effects of increased cellular H_2O_2 concentrations, both at rest and during stimulation, along with the activation of signalling pathways by contraction (e.g., MAPK, AMPK, CaMK) are likely to play a role in the changes in mRNA expression seen in this study. This may contribute to the upregulation of genes associated with angiogenesis, fatty acid metabolism, antioxidant potential, and glucose uptake. The increased activity of these signalling pathways is not dependent on GSH or are sufficient to override the lack of GSH.

Conclusion

GSH depletion did not impair gene expression following ES. Although changes were seen in the absence of electrical stimulation, these may be due to acute redox signalling. Further investigation into the specific mechanisms is required to identify potential contributing factors, such as altered levels of H_2O_2 and MAPK, AMPK and CaMK signals which arise from exercise, known to modulate gene expression.

Key words: *Glutathione, skeletal muscle, exercise adaptation, electrical stimulation, myotubes, ROS, oxidative stress, BSO*

Acknowledgements

There are many people I would like to acknowledge that have enabled me to complete this thesis. Thank you to my supervisors Martin Dickens and Kaio Vitzel for the countless hours you both have spent with me in the lab and reviewing my work. Your continued dedication and support has been greatly appreciated and this would not have been possible without you.

I would like to thank all my family, friends and loved ones for their continued support throughout these years. To all my friends who have been there when I needed a break and my partner Maxine who has ensured that I am fed and watered through long hours at my desk. A special thanks to Ignas Palamarcukas for all of your hours and hours of help in the lab. Finally, I would like to express many thanks to my parents – they have supported me and motivated me. I would not have been able to do this without their continued support.

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List of Abbreviations

ACC	Acetyl-CoA Carboxylase
ADaM	Allosteric Drug and Metabolite
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AMPK	AMP-activated Protein Kinase
AP-1	Activator Protein-1
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
BSO	Buthionine Sulphoximine
CaMK	Calcium/Calmodulin Dependent Protein Kinase
cDNA	Complementary DNA
CPT-1	Carnitine Palmitoyltransferase I
CREB	cAMP-Response Element Binding Protein
DHPR	Dihydropyridine Receptor
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ERK	Extracellular Signal-regulated Kinase
ES	Electrical Stimulation
FBS	Fetal Bovine Serum
γ-GCS	Gamma-glutamylcysteine Synthetase
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSHEE	Glutathione Ethyl Ester
GSSG	Glutathione Disulphide; Oxidised Glutathione
GST	Glutathione S-Transferase
GSTM	Glutathione S-Transferase Mu
GSTP	Glutathione S-Transferase P
HDAC	Histone Deacetylase
HIF	Hypoxia-inducible factor

HK	Hexokinase
HPRT	Hypoxanthine-guanine Phosphoribosyl Transferase
HRP	Horseradish Peroxidase
JNK	c-Jun NH2-terminal Kinase
LKB1	Liver Kinase B1
LPL	Lipoprotein Lipase
MAPK	Mitogen-Activated Protein Kinase
MEF2	Myocyte Enhancer Factor 2
MEKK1	MAPK/ERK Kinase Kinase 1
MHCI	Myosin Heavy Chain I
mRNA	Messenger RNA
MyoD	Myoblast Determination Protein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor kappa-light-chain-enhancer of Activated B cells
NOX	NADPH Oxidase
NRF	Nuclear Respiratory Factor
ODDD	Oxygen dependent Degradation Domain
PGC-1α	Peroxisome Proliferator-activated Receptor gamma Co-activator 1α
PHD	Prolyl Hydroxylase Domain-containing Enzymes
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PLA2	Phospholiapse A2
PPAR	Peroxisome Proliferator-activated Receptor
PUFA	Polyunsaturated Fatty Acids
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
SAPK	Stress-activated Protein Kinase

SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SIRT1	Sirtuin 1
SOD	Superoxide Dismutase
SSA	5-dihydrate Sulfosalicylic Acid
T2D	Type 2 Diabetes
Tfam	Mitochondrial Transcription Factor A
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau E3 ubiquitin-ligase
VLDL-TG	Very-low-density Lipoprotein-triglycerides
XO	Xanthine Oxidase

1. Introduction

1.1 Background

Due to its mass, endocrine function, metabolic capacity, and role in movement, human skeletal muscle is important for protein turnover as well as the storage and metabolism of glucose and fatty acids. Consequently, skeletal muscle has a significant impact on the greater metabolic and functional health of the whole body (Kim & Kim, 2020). Accounting for ~40% of total body weight, muscle receives ~25% of cardiac output at rest, and any irregularities in function can lead to much wider negative implications. Pathologies affecting muscle can a have significant effect on an individual's mobility and metabolic health (Baar & Hargreaves, 2011). Consequently, skeletal muscle mass is a key indicator for health and longevity, with loss of muscle and altered energy metabolism being linked to numerous disease conditions, such as diabetes and cancer. Ranging from neuromuscular to metabolic diseases, the most common causes of muscular disfunction are suggested to be abnormal ATP production, a lack of Ca^{2+} homeostasis and mitochondrial disfunction (Mukund & Subramaniam, 2020). These imbalances in energy metabolism also trigger muscle wasting, causing muscle mass loss. Along with the function of the muscle as a whole, the activity of key enzymes involved in glucose homeostasis, fatty acid metabolism, mitochondrial biogenesis and many other important metabolic processes have been shown to be important biomarkers for muscular disease progression (Santacatterina et al., 2015).

Skeletal muscle is one of the most plastic tissues in the human body allowing it to adapt to stressors and stimuli, responding with physiological and biochemical changes within the muscle (Baldwin & Haddad, 2002; Chromiak & Antonio, 2008; Fluck & Hoppeler, 2003). Proteomic screens showed that 562 proteins become phosphorylated in human skeletal muscle following exercise, suggesting that an extensive range of signalling pathways are affected by contraction (Hoffman et al., 2015). Chemical changes along with mechanical stress within the cell during and following accumulative bouts of exercise triggers a wide range of signalling pathways leading to changes in enzyme activity and gene expression. During exercise the production of reactive oxygen species (ROS) are significantly increased throughout the muscle cell (Frontera & Ochala, 2015; Packer, Cadenas, & Davies, 2008). These highly reactive molecules act as both destructive oxidisers as well as important signalling molecules (Allen & Tresini, 2000; Bauer, Elsen, & Bird, 1999; Jackson, Stretton, & McArdle, 2020; Le Moal et al., 2017).

Chronic elevation of ROS levels can lead to extensive cell damage and is an important factor in diseases, such as cancer and diabetes (Liguori et al., 2018). In order to maintain ROS homeostasis, skeletal muscle cells have an extensive antioxidant system which controls the redox state of the cell. Antioxidant capacity increases in response to exercise-derived ROS. Glutathione (GSH), a three amino acid peptide and the most abundant antioxidant in muscle, helps maintain ROS homeostasis (Forman, Zhang, & Rinna, 2009). Recent research suggests that GSH may also play a role in the activation of enzymes and pathways known to change following exercise. Therefore, oxidised GSH may affect the magnitude of changes in gene expression and enzyme activity by S-glutathionylation and modulation of cellular redox status in a way that changes the physiology of the muscle cell. These adaptations can not only improve physical performance but can improve overall health and reduce the incidence of disease (Booth, Laye, Lees, Rector, & Thyfault, 2008).

A more in-depth understanding of the biological processes involved in muscle adaptation may lead to the ability to manipulate these processes in order to maximise beneficial responses to exercise. Current evidence suggests that these adaptations occur through multiple signalling pathways, such as AMPK and MAPK, activated during and following exercise. When the activity of these pathways is increased or changed, then changes in the expression of genes involved in biological processes can occur, including mitochondrial biogenesis, fibre type switching, energy metabolism and antioxidant capacity. GSH is suggested to play a role in affecting the magnitude of these changes. Further, total GSH levels and the ratio between oxidized and reduced glutathione (GSSG/GSH) may be an important indicator for some medical conditions while also being a useful target for intervention and utilised in conjunction with other interventions.

The following literature review will present the current knowledge on the molecular adaptations of skeletal muscle to exercise, focusing on improved glucose and fatty acid metabolism, mitochondrial biogenesis, fibre-type switching, angiogenesis and improved antioxidant potential. Emphasis is placed on GSH and its role as an antioxidant as well as the role it may play in muscle adaptations. This review aims to outline the potential changes in signalling pathways, enzyme activity and gene expression which GSH may modify.

1.2 Objective and Hypothesis

The aim of this study is to evaluate the role of endogenous GSH in skeletal muscle adaptation to contraction induced by electrical stimulation (ES) *in vitro*. We hypothesise that GSH plays a role in contraction-induced gene expression in C2C12 myotubes. Measurable differences in gene expression will be seen following ES of differentiated C2C12 myotubes when comparing GSH depleted cell groups to control groups. GSH depletion will impair signalling pathways involved in skeletal muscle adaptation and attenuate induced increases in gene expression.

1.3 Specific objectives

1.3.1 Obtain a time course of C2C12 myotube gene expression at baseline and after electrical stimulation (ES):

- The expression of genes will be measured at three timepoints, immediately following stimulation, one hour after and three hours after stimulation, as well as for a non-stimulated control. These time points were designed to display the expression of targeted genes known to be involved in muscle adaptation. Genes of interest are known to peak around the specific time points used in this study (Bickel et al., 2005; Pilegaard et al., 2002; Yang, Creer, Jemiolo, & Trappe, 2005). The target genes cover various aspects of adaptation including oxidative stress response, mitochondrial biogenesis along with fatty acid and glucose metabolism.

1.3.2 Measure ROS production during the first three hours of electrical stimulation:

- This was done through the measurement of H_2O_2 released by C2C12 myotubes and provides an indicator as to the overall ROS production of the cell and levels of oxidative stress which the cells are under.

1.3.3 Assess GSH content of the cells following stimulation and at baseline:

- In order to determine the success of the GSH depletion treatment as well as assessing GSH production in response to stimulation.

1.3.4 Analyse the survival rate of cells:

- Asses the percentage of cells still viable following ES and/or GSH depletion to ensure survival rates of ~95%.

1.3.5 Measure the effect of GSH depletion on contraction-stimulated gene expression

- Determine if GSH depletion has any effect on the expression of genes related to adaptation following contraction and, if so, to what extent are they effected.

2. Literature Review

2.1 Signals from Muscle Contraction Trigger Exercise Adaptation

Skeletal muscle mass is a key indicator for health and longevity (McLeod, Breen, Hamilton, & Philp, 2016). Muscle atrophy and impaired metabolism has been linked to increased risk of cancer and diabetes. It may also lead to reduced mobility, further increasing the incidences of comorbidities such as cardiovascular disease and causing a loss of independence and quality of life. Exercise promotes beneficial changes in both muscle mass and metabolism, potentially leading to protective effects against a range of pathological conditions.

Research has shown a distinction in the way which skeletal muscle adapts to endurance training in comparison to resistance training. The signalling pathways activated and physiological adaptations which follow these different training stimuli cause different responses. Resistance/strength training primarily stimulates processes such hypertrophy and protein synthesis and turnover resulting in greater muscle volume and strength (Fry, 2004). In contrast, endurance training elicits a range of metabolic adaptations such as increased mitochondrial content and respiratory capacity leading to increased tolerance to fatigue (Holloszy, 1967). These adaptations to exercise are brought about through changes in gene expression within the muscle. Over time, the accumulative effect of multiple exercise bouts results in different genes being activated and supressed, changing metabolic and physiological properties of the muscle and allowing it to better cope with similar stresses in the future.

2.1.1 ATP

ATP is required for many actions within skeletal muscle, with large amounts being consumed by the actin-myosin cross-bridge cycle and ion pumps. The amount of ATP required by the cell during exercise is dependent on the intensity and duration of contraction. Similarly, the way in which ATP is produced and levels are maintained varies depending on the same factors that affect ATP requirement, primarily intensity and duration of contraction (Frontera & Ochala, 2015). While the phosphagen pathway (phosphocreatine and adenylate kinase) restores ATP stores for a very short period (seconds), the oxidative metabolism of lipids and carbohydrates are an important source of ATP production during short-term and extended exercise (Spriet & Watt, 2003). The contribution of carbohydrates to ATP production increases as energy expenditure and exercise intensity increase (Sahlin, Tonkonogi, & SÖDerlund, 1998), while glycogen phosphorylase regulates glycogen breakdown, one of the main systems of anaerobic ATP production within skeletal muscle (Komoda & Matsunaga, 2015). Muscle glycogen is the predominant source of carbohydrates with extracellular glucose utilisation increasing as the exercise period extends.

Another key source of fuel is fatty acids. They provide their most significant contribution to energy production at lower levels of contraction (around 60% $\dot{V}O_{2\ max}$ exercise intensity) and as exercise progresses. Long duration moderate exercise at ~65% $\dot{V}O_{2\ max}$ is primarily fuelled by oxidative ATP production from both fatty acid and carbohydrates while yields from amino acids are significantly lower (5-15% of ATP production) (Baker, McCormick, & Robergs, 2010). As the period of moderate exercise extends over several hours, there is a measurable shift in metabolism towards increased lipid oxidation, while carbohydrate oxidation and the contribution of muscle glycogen to ATP production decreases (Hargreaves & Spriet, 2020). During exercise, the utilisation of ATP can exceed production, causing ATP depletion which is a cause of peripheral fatigue.

5' AMP-activated protein kinase (AMPK) is an enzyme which plays a key role in energy homeostasis within cells acting as a sensor of low intracellular ATP levels (Herzig & Shaw, 2018). AMPK responds to metabolic stress by switching off anabolic pathways, reducing ATP consumption, while switching on catabolic pathways therefore increasing ATP production. AMPK works over both the short and long-term, changing enzyme activity to affect metabolism quickly, while also modifying gene expression to bring about longer-term effects. There is now an extensive body of research surrounding AMPK activation and its role in maintaining energy homeostasis within cells (Carling, Mayer, Sanders, & Gamblin, 2011; Hardie, Carling, & Gamblin, 2011; Hardie, Ross, & Hawley, 2012).

2.1.2 Depolarisation (Calcium Signalling)

Skeletal muscle depolarisation triggers the coupling of dihydropyridine receptors (DHPRs), located on the muscle cell surface, with ryanodine receptors (RyR) of the sarcoplasmic reticulum, releasing Ca^{2+} from within the sarcoplasmic reticulum into the intracellular space (Lanner, Georgiou, Joshi, & Hamilton, 2010). There are three different isoforms of ryanodine receptor with RyR1 being the major form found in skeletal muscle (Inui, Saito, & Fleischer, 1987; Takeshima et al., 1989). The interaction between RyRs and DHPRs after membrane depolarisation causes a conformational change in the RyR allowing the rapid release of calcium into the cytosol and increasing cytosolic calcium concentrations by ~100 fold (Nelson et al., 2013; Rios & Brum, 1987). At this high concentration calcium binds to troponin C on the thin filament of the muscle fibre and contraction is activated. Along with contraction, Ca^{2+} also regulates a wide range of molecular processes, through calcium binding proteins, involved in cell differentiation, protein metabolism and gene expression (discussed later in the Literature Review) (Berchtold, Brinkmeier, & Müntener, 2000). The levels of intracellular calcium are monitored and in part controlled by the sarcoplasmic reticulum (SR). Following depolarisation, RyR/DHPR interaction, the release of calcium into the cytosolic space, and the activation of downstream signalling pathways, the calcium is then cleared from the cytosol. The primary pathway for Ca^{2+} reuptake in skeletal muscle is into the sarcoplasmic reticulum via sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) (Rossi & Dirksen, 2006). This pump controls muscle relaxation following contraction and limits the activation of any downstream signalling pathways which are activated by the acute increase in cytosolic calcium concentration. This calcium transient is a major second messenger in muscle, activating pathways such as CaMK and calmodulin. These pathways contribute to adaptations following exercise, including the upregulation of angiogenesis and mitochondrial biogenesis, which are discussed in detail later in the review.

2.1.3 Mechanical Stress

Skeletal muscle responds to mechanical stress during contraction. The extracellular matrix allows the force of contraction to reach the surface of the cell and interact with integrins (Sakamoto & Goodyear, 2002). Activated integrins have been shown to mediate a wide range of molecular responses through an integrin linked kinase leading to activation of the Mitogen-Activated Protein Kinase (MAPKs), c-Jun NH2-terminal kinase (JNK) and Extracellular Signal-Regulated kinase (ERK) (Miyamoto et al., 1995). Signals from within the cytoskeleton are also crucial for the transduction of mechanical-related stimuli within skeletal muscle. Costameres are composed of Z-line associated proteins that play a key role in transmitting mechanical signals between the sarcomere, extracellular matrix, and sarcolemma. These structures provide a two-way signalling pathway between the extracellular matrix and the Z-line, allowing for the transduction of external mechanical stress signals to a range of intracellular signalling pathways (Peter, Cheng, Ross, Knowlton, & Chen, 2011).

Mechanical signals are required to maintain and develop skeletal muscle. Mechanotransduction is the processes that allows signals such as physical shape changes in the muscle to be converted into intracellular biochemical signals (Burkholder, 2007). Muscle adaptation to exercise relies on the ability for the resulting stimuli to trigger biochemical changes within the cell. Conversely, a lack of stimuli can cause muscle wasting and a decrease in muscular function. One of the key players in mechanotransduction in skeletal muscle is focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase which directly translates the mechanical stress placed on muscle transmitted through the cytoplasmic membrane via integrins (Graham, Gallagher, & Cardozo, 2015). When the levels of FAK expression or phosphorylation within a muscle changes, this directly effects protein synthesis, cell growth, anti-apoptotic pathways and mechanotransduction (Carson & Wei, 2000). The most prominently affected of these is the Phosphoinositide 3-kinase (PI3K) pathway which includes the mammalian target of rapamycin (mTOR) and Akt/protein kinase B (PKB). Together these signalling pathways act as master regulators of protein synthesis and cell size. PI3K pathways are also involved in energy metabolism within skeletal muscle, helping to regulate insulin-stimulated glucose uptake (Huang, Khoe, Ilic, & Bryer-Ash, 2006). Although the molecular details of the mechanotransduction pathways affecting skeletal muscle adaptation are not yet fully understood, it has been suggested that physical stresses can directly and rapidly effect gene expression leading to significant physiological and biochemical changes to the muscle. Further research is required to help uncover the role mechanotransduction plays in adaptation as well as muscle-related diseases (Olsen, Nicoll, & Fry, 2019).

2.1.4 Reactive Oxygen Species

Free radicals are atoms/molecules that contain one or more unpaired valency electrons (Powers, Ji, Kavazis, & Jackson, 2011). The nature of this unpaired electron causes free radicals to be unstable and reactive. Radicals are produced in cells through a multitude of different reactions constantly taking place as a part of normal physiological function. Reactive oxygen species (ROS) is the general term given to oxygen based free radicals as well as other reactive derivatives of oxygen (Powers et al., 2011). Two of the main ROS produced in cells are superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Their production increases during exercise, when they act modulating contractile function and as signalling molecules (Droge, 2002). These molecules and the signalling pathways they are involved in are detailed further below.

2.2 Main Sources of ROS in the Muscle

Reactive oxygen species (ROS), such as $O_2^{\bullet-}$ and $H_2O_{2,}$ are produced in all eukaryotic cells. Skeletal muscle is no different with ROS production occurring during a wide range of physiological processes. The mitochondrial electron transport chain is a key producer of ROS in muscle (Powers & Jackson, 2008). Complexes I and III of the electron transport chain have been shown to be the primary producer of ROS in skeletal muscle, with levels of production increasing by up to 187% from baseline during exercise (Saborido, Naudi, Portero-Otin, Pamplona, & Megias, 2011). Although mitochondria have long been considered the main source of ROS during exercise this has, more recently, been challenged. Studies show that mitochondria produce significantly less ROS than previously thought during intense aerobic contractile activity (Di Meo & Venditti, 2001; Herrero & Barja, 1997; Mason, Morrison, McConell, & Wadley, 2016; Merry & Ristow, 2016). Other significant sources of intracellular ROS include NADPH oxidases (NOXs), phospholipase A2 (PLA2), and xanthine oxidase (XO) (Gomez-Cabrera et al., 2005; Piao et al., 2005; Powers & Jackson, 2008; Powers, Nelson, & Hudson, 2011).

2.2.1 NADPH Oxidases

The NADPH oxidase (NOX) protein family consists of five members NOX1-5. They have both electron-transporting and catalytic functions and generate superoxide. NADPH and oxygen are converted into $O_2^{\bullet-}$ via these partially membrane-bound protein complexes. In skeletal muscle only the NOX2 and 4 isoforms are present, located on transverse tubules, the sarcolemma, and the sarcoplasmic reticulum (Abrigo et al., 2018).

NOX2 produces $O_2^{\bullet-}$ ($NADPH+2O_2 \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+$), while NOX4 produces both $O_2^{\bullet-}$ and H_2O_2 (Nisimoto, Diebold, Cosentino-Gomes, & Lambeth, 2014; Serrander et al., 2007). Some $O_2^{\bullet-}$ produced by NOX is converted into H_2O_2 by superoxide dismutases (SODs) ($2H^+ + O_2^{\bullet-} \rightarrow H_2O_2 + O_2$). The overactivity of NOX within skeletal muscle has been shown to lead to atrophy (Cunha et al., 2017). This effect was significantly reduced by aerobic exercise which caused reductions in NOX2 activity resulting in decreased ROS. Chronic excessive ROS, including that produced by NOX, has also been directly linked to progressive muscle damage and aging (Mansouri et al., 2006). The H_2O_2 produced by SODs from the $O_2^{\bullet-}$ of NOX plays an important role in redox signalling within skeletal muscle. The ability for H_2O_2 to diffuse outside of membranes allows it, along with its relatively long half-life, to interact with a range of molecules and affect a range of physiological processes from enzyme activity to gene expression. This diffusion allows for the measurement of H_2O_2 produced in cells by assaying the concentration of H_2O_2 in samples of extracellular fluid. During both rest and exercise NOX2 and 4 have been shown to contribute to ROS production to a larger extent than mitochondria (Cherednichenko et al., 2004; Espinosa et al., 2006; Hidalgo, Sanchez, Barrientos, & Aracena-Parks, 2006; Sakellariou et al., 2013; Steinbacher & Eckl, 2015). Specifically, the activity of NOX2 increases during and following exercise, increasing the levels of ROS within the muscle, while NOX4 is constitutively active.

The difference in ROS production between fibre types was originally suggested to be due to the differences in mitochondrial content of each fibre. However, more recent research has shown that the mitochondria are not the primary site of ROS production in skeletal muscle and that differences in ROS production may be due to other factors such as varying NOX levels. Type I oxidative fibres have been shown to have higher activity of the ROS producing NOX enzymes along with increased NOX2 and 4 mRNA levels (Loureiro et al., 2016). These variations may account for the difference in ROS levels between muscle fibre types beyond mitochondrial density. Type I fibres also showed higher levels of antioxidant enzyme activity such as glutathione peroxidase (GPx) which will be discussed further later in the review.

2.3 Uncontrolled ROS in Skeletal Muscle

In the normal physiological state, ROS within skeletal muscle are kept at a level where they act as signalling molecules without causing significant oxidative damage. But when the production of ROS increases or antioxidant levels decrease, the antioxidant-oxidant balance is no longer maintained, resulting in a wide range of oxidative damage throughout the muscle. Acute increases in ROS may cause no significant damage to the cell and is in fact required for the activation of some signalling pathways. Chronic elevation of ROS levels is likely to lead to disrupted redox signalling and cellular metabolism along with misregulation and damage of cellular constituents (Lushchak, 2011). Hydrogen peroxide (H_2O_2) is a nonradical weak oxidant with a relatively long half-life in comparison to other ROS produced within muscle. The ability of H_2O_2 to diffuse across cell membranes, means it works well as a signalling molecule. Other ROS, such as the highly reactive $O_2^{\bullet-}$ produced by NOX and the mitochondria, react immediately with surrounding molecules such as nitric oxide. This reaction results in the production of the anion peroxynitrite ($ONOO^-$), another signalling molecule utilised in muscle. Although useful for signalling, excessive levels of peroxynitrite within a cell leads to significant oxidative damage and apoptosis (Lin, Xue, Sun, & Wong, 1997).

High concentrations of ROS can cause lipid peroxidation in cell membranes, triggering a chain reaction which can lead to the degradation of cell integrity. This is discussed in more detail later in this Literature Review. The reactive hydroxyl radical ($OH^\bullet$) affects DNA, damaging pyrimidine bases and deoxyribose, potentially causing mutations and leading to numerous pathological conditions, such as cancer (Packer et al., 2008). These hydroxyl radicals can be formed via metal-catalysed reactions of H_2O_2 such as the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^\bullet + OH^-$). Another common reaction in skeletal muscle is the Haber-Weiss reaction which sees iron or copper being held in a reduced state by $O_2^{\bullet-}$ allowing the formation of $OH^\bullet$ from H_2O_2 ($O_2^{\bullet-} + H_2O_2 \rightarrow OH^\bullet + OH^- + O_2$). ROS within the cell also react with proteins, targeting various amino acids and forming protein carbonyls. These effects can result in mitochondrial disfunction along with apoptosis and necrosis (Steinbacher & Eckl, 2015). Contractile disfunction and reduced force generation caused by oxidative damage contribute to muscle atrophy, aging, as well as a range of other pathophysiological conditions (Mecocci et al., 1999). The combination of these forms of oxidative damage over time are significant contributors to conditions such as diabetes, cancer, and aging. Therefore, the reduction of this damage by antioxidants can help reduce the rates of these conditions in humans (Blokhina, Virolainen, & Fagerstedt, 2003; Kurutas, 2016; Liguori et al., 2018).

2.4 Impaired Exercise Response due to Antioxidants

Antioxidant loading, the supplementation of high levels of antioxidants prior to physical activity, may benefit training and performance if the stress exerted on the muscle is enough to elevate ROS levels to a point where they begin to impair function during exercise (Bentley, Ackerman, Clifford, & Slattery, 2015; Braakhuis & Hopkins, 2015). However, antioxidant supplementation has also been suggested to have no effect on, or even potentially increase, the incidence of disease in humans (Ristow, 2014). This is due to the non-targeted and non-specific antioxidants, such as vitamins C and E, which are typically used in human intervention studies. These antioxidants aim to scavenge ROS throughout the body in comparison to the endogenous cellular redox defences which are highly compartmentalised and regulated by complex signalling pathways (Merry & Ristow, 2016; Ristow, 2014). The supplementation or over supplementation of exogenous antioxidants does not specifically target the potentially harmful ROS but all ROS including those which play an important role in the signalling required for muscle adaptation to exercise.

A range of studies have shown that antioxidants attenuate muscle adaptations, such as mitochondrial biogenesis, following exercise (Gomez-Cabrera et al., 2005; G. D. Wadley, Nicolas, Hiam, & McConell, 2013). Animal studies found that antioxidants blunted the increase in markers of mitochondrial biogenesis, such as peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) and nuclear respiratory factor 1 (NRF1), but without measurable effects on mitochondrial biogenesis. There appears to be decreased signalling in animals treated with antioxidants yet no direct impact on mitochondrial number or mitochondrial mass (Abadi et al., 2013; Meier, Renga, Hoppeler, & Baum, 2013). These findings suggest that although antioxidants do affect the signalling pathways related to mitochondrial biogenesis following exercise, there is some redundancy in the signalling which can allow for biogenesis to take place even when ROS signalling has been depleted (Merry & Ristow, 2016). The effect which antioxidants have on skeletal muscle biogenesis is likely to be affected by a range of factors including the timing and duration of supplementation as well as the type of antioxidants and the exercise protocol.

2.5 Main Antioxidant Molecules/Systems in Muscle

2.5.1 Superoxide Dismutases

Superoxide dismutases (SODs) are enzymes which catalyse the dismutation of $O_2^{\bullet-}$ into H_2O_2 (Le Moal et al., 2017). There are three isoenzymes SOD1, 2, and 3. These enzymes require redox-active transition metals such as copper and zinc, in their active site in order to catalyse dismutation (Powers et al., 1994). SOD1 is located in the mitochondrial intermembrane space and the cytosol, SOD2 is also found in mitochondria, while SOD3 is extracellular (Folz & Crapo, 1994; Godenschwege et al., 2009; Nagahisa, Okabe, Iuchi, Fujii, & Miyata, 2016).

2.5.2 Catalase

Catalase catalyses the dismutation of H_2O_2 producing H_2O and O_2. Located primarily in the peroxisome, catalase has four subunits and requires iron as a cofactor in its reaction (Christie & Steward, 1979; Van Der Vliet & Janssen-Heininger, 2014). Studies have shown that decreased catalase activity and therefore increased oxidative stress in skeletal muscle is associated with the age-related loss of muscle mass (Sullivan-Gunn & Lewandowski, 2013).

2.5.3 Glutathione

Glutathione (GSH) is a tripeptide (cysteine, glycine, and glutamic acid) that is the most abundant antioxidant molecule in skeletal muscle and functions to maintain intracellular redox status and prevent oxidative damage (Pizzorno, 2014). GSH is primarily located in the cytosol where it is involved in a wide range of reactions which help buffer muscle oxidant levels. It is a substrate for glutathione peroxidases (GPxs) which catalyse the reduction of H_2O_2 by GSH to H_2O and glutathione disulphide (GSSG) ($2GSH + H_2O_2 \rightarrow GS–SG + {}_2H_2O$) (Brigelius-Flohe, 1999; Forman et al., 2009). The resulting GSSG is then reduced by glutathione reductase (GR) back to GSH using the reducing power of NADPH ($GS–SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$), as shown in Figure 1. Type I skeletal muscle fibres contain higher levels of GSH in comparison to type II fibres as their oxidative/aerobic metabolism produces higher levels of ROS (Leeuwenburgh et al., 1997b). GPx1 is a particularly important member of the GPx family found in skeletal muscle within the cytoplasm and mitochondria, playing a significant role in reducing the accumulation of H_2O_2 in the cell (Brigelius-Flohé & Maiorino, 2013). Its role as an antioxidant enzyme also means that it contributes to the control of downstream redox-sensitive pathways.

GPx3 also works to maintain the antioxidant balance of skeletal muscle, scavenging extracellular hydrogen peroxide in the presence of reduced GSH (Takebe et al., 2002). Known as a plasma GPx, GPx3 also catalyses reactions with organic and lipid hydroperoxides alongside hydrogen peroxide (Maddipati & Marnett, 1987).

The involvement of GSH in physiological processes is often studied by inhibiting its production thereby depleting the endogenous GSH within the muscle. Glutathione is synthesised in muscle cells through two ATP dependant reactions. The first of which involves the production of gamma-glutamylcysteine from L-glutamate and cystine, carried out by gamma-glutamylcysteine synthetase (γ-GCS). Buthionine sulfoximine (BSO) is an amino acid which directly and irreversibly inhibits γ-GCS, therefore depleting GSH levels within the cell. It does this by acting as an analogue of the transition-state in the γ-GCS reaction (Ardite, Barbera, Roca, & Fernández-Checa, 2004). This method of depleting GSH within muscle is used in the current study to investigate the antioxidant`s involvement in the regulation of genes associated with muscle adaptation following contraction.

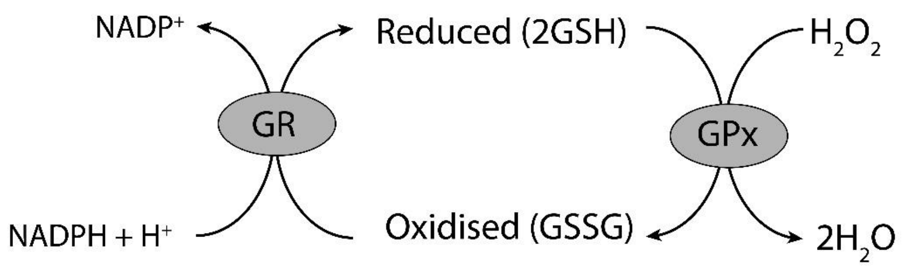

Figure 1. The GSH antioxidant system. Within skeletal muscle, glutathione peroxidases (GPx) catalyse the reduction of H_2O_2 by oxidising GSH and producing $2H_2O$ and GSSG. This neutralises the potentially harmful H_2O_2 reactive oxygen species. The oxidised GSSG is able to be recycled via a reduction by glutathione reductase (GR) and donation of a electrons from NADPH + H^+. This reaction results in 2GSH and $NADP^+$. The GSH is then able to continue this cycle and provide its antioxidant function within the muscle.

2.5.4 Glutaredoxins and Peroxiredoxin

Along with GSH and GPx, glutaredoxins play an important part in the maintenance of the oxidative state of cells. Glutaredoxins are redox enzymes which deglutathionylate proteins, being glutathionylated in the process (Fernandes & Holmgren, 2004).

Glutaredoxins are able to react with other oxidised proteins, restoring them to their reduced form by utilizing the reducing power of GSH, regenerating glutaredoxin while reverting the GSH to its oxidised form GSSG (Lillig, Berndt, & Holmgren, 2008). GSSG is then reduced through the reaction with GR and NADPH as previously described. Glutaredoxins provide additional antioxidant capability to skeletal muscle and are an important part of the redox regulatory mechanism.

Peroxiredoxins are a family of potent antioxidant proteins present within skeletal muscle, located in the cytosol, endoplasmic reticulum, and mitochondria. These thiol peroxidases catalyse the reduction of peroxides and have been shown to be play a key role in linking increased H_2O_2 production during exercise to the physiological adaptations which follow (A. J. Wadley, Aldred, & Coles, 2016). The resulting sulfenic acid produced from the reduction of H_2O_2 by peroxiredoxin may act as a signalling molecule, able to form mixed disulphide bonds with transcription factors. This reaction may allow peroxiredoxin to mediate signals produced by exercise-induced oxidants and translate them into effects on downstream transcription factors. Currently, p38 MAPK is one of the primary signalling pathways known to be sensitive to phosphorylation by peroxiredoxin-mediated signalling via apoptosis signalling kinase 1 (ASK1) (Jarvis, Hughes, & Ledgerwood, 2012). Peroxiredoxin can also be inactivated by glutathionylation, resulting in a build-up of H_2O_2 in specific cellular regions, therefore redirecting the H_2O_2 signal (Kil et al., 2012).

2.5.5 Vitamins C and E

Vitamin C can minimise damage from radical chain reactions within the cell caused by ROS. Lipid peroxidation is the oxidative degradation of lipids which commonly occurs within skeletal muscle. Due to many ROS producing enzymes being located within lipid bilayers along with the relative weakness of the bisallylic C-H bond in comparison to other C-H bonds, lipids are one of the primary targets of ROS in muscle. The reaction targeting lipids is initiated by a free radical attacking a lipid-hydrogen (LH) compound which is converted into a lipid peroxyl radical ($LOO^{\bullet}$), which then converts another LH into a carbon-centred lipid radical ($L^{\bullet}$) (El-Aal, 2012). This process can initiate a chain reaction of lipid peroxidations, leading to the accumulation of chemically unstable lipid hydroperoxides (LOOH) within the muscle. Lipid peroxidation is known to contribute to an increased progression of many chronic diseases, such as cardiovascular disease (Mattson, 2009), diabetes (Traverso et al., 1998) and neurodegenerative disease (Butterfield, Yatin, Varadarajan, & Koppal, 1999).

Both vitamins C and E play a role in diminishing the effects of lipid peroxidation on muscle. The $LOO^{\bullet}$ produced during lipid peroxidation reacts 1000-times faster with vitamin E than it does with polyunsaturated fatty acids (PUFA) causing it to be preferentially oxidised (Buettner, 1993). This allows vitamin E to react with the radical $LOO^{\bullet}$ producing the oxidised Vit $E\text{-}O^{\bullet}$ and the corresponding LOOH. At this stage vitamin C is able to donate an electron to the Vit $E\text{-}O^{\bullet}$, oxidising the vitamin C while returning vitamin E to its reduced state (Neuzil, Witting, & Stocker, 1997). This series of reactions has been termed as ‘vitamin E recycling’, and the constant renewal of the antioxidant function of vitamin E through its reaction with other antioxidants creates an antioxidant network within the muscle (Figure 2).

Along with its reaction with oxidised vitamin E, vitamin C can also scavenge free ROS by donating electrons to potentially harmful ROS. In this case and that involving vitamin E recycling, oxidised vitamin C is a product of these reactions. There are multiple enzymatic pathways which allow vitamin C to be returned to its reduced state and once again take part in the antioxidant network within the muscle (Padayatty et al., 2003). In humans the reduction back to ascorbic acid is only partial as some is lost to hydrolysis.

As vitamin C is not synthesised within the body, supplementation may have a protective effect against oxidative damage by lipid peroxidation. A range of studies have shown vitamin C supplementation decreased lipid peroxidation levels as well other biomarkers of oxidative stress (Harrison, Meredith, Dawes, Saskowski, & May, 2010; Kuiper, Bruno, Traber, & Stevens, 2011; Tveden-Nyborg & Lykkesfeldt, 2009).

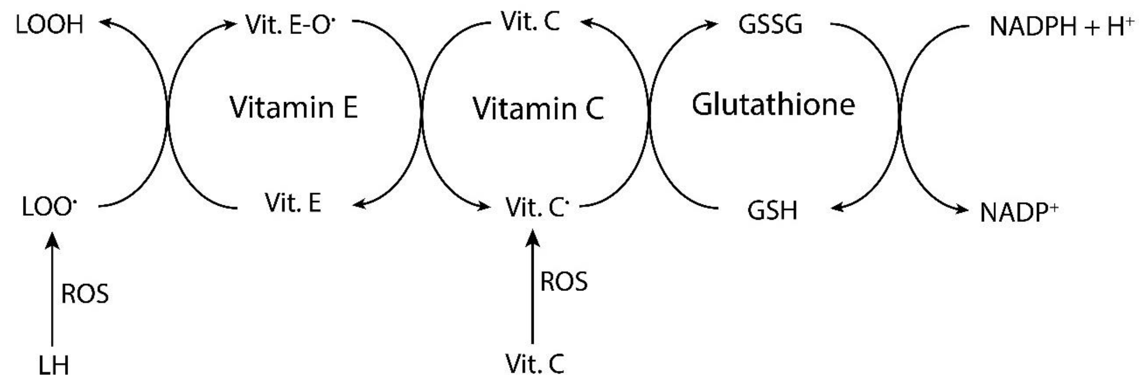

Figure 2. The antioxidant activity of vitamins E and C. Lipid peroxidation is a major form of oxidative damage caused by ROS. It begins with the oxidation of a lipid substrate (LH) into a lipid peroxyl radical (LOO•) by a ROS. This lipid radical can go onto convert another LH into LOO•, itself converting to a lipid hydroperoxide (LOOH). This begins a chain reaction which can work its way throughout the lipid membrane of a muscle cell causing extensive damage to the function and structural integrity of the membrane. Vitamin E works as an antioxidant which is preferentially oxidised by LOO• over lipid molecules. This breaks the chain of lipid peroxidation helping to prevent further damage. The vitamin E is oxidised (Vit. E-O•). Vitamin C also has antioxidant capabilities allowing it to be oxidised by the reactive Vit. E-O• into Vit. C•, effectively passing on the donated electron, returning vitamin E to its base state where it can again react with LOO•. Vitamin C can also be directly oxidised by other ROS also producing Vit. C•. GSH then allows vitamin C to be reduced back to its base state and be reused through its own oxidation to GSSG. As previously described GSSG is reduced to GSH via NAD(P)H in a reaction catalysed by glutathione reductase (GR).

2.5.6 Exogenous Antioxidant Molecules

There are many molecules with antioxidant properties which are not produced within the cell. These can be generally classified into vitamins, trace elements, and phytochemicals. One of the most prominent being the aforementioned vitamin E, which refers to four tocopherols and four tocotrienols (α, β, δ, γ) primarily found in the plasma membrane in skeletal muscle (Brigelius-Flohe & Traber, 1999). These molecules react with oxidants in a process which encompasses GSH, the products of this reaction are then further reduced by vitamin C (Khor, Abdul Karim, Ngah, Yusof, & Makpol, 2014). Increased local vitamin E levels have been associated with protective effects against exercise-induced oxidative stress (Meydani, Fielding, & Martin, 1998).

A range of trace elements including copper, zinc, and manganese play a protective role in skeletal muscle cells. These are typically used as cofactors in redox reactions as previously mentioned regarding SODs. Studies have shown that these trace elements can be equally as important as other antioxidant molecules in maintaining the cellular redox status and minimising oxidative damage (Gaetke & Chow, 2003; Oteiza, 2012).

2.6 ROS Increase in Muscle During Exercise and Antioxidant Response

Skeletal muscle is one of the most plastic and responsive organs in regards to adaptation to exercise (Bassel-Duby & Olson, 2006). Following exercise, the muscle responds and adapts in a way which allows it to better cope with future exercise stress. The positive effects of this adaptation extend beyond benefits to physical exercise also allowing muscle to better deal with similar stressors such as those related to disease development (Merry & Ristow, 2016). One of the main stressors present during exercise is an increase in ROS within muscle (Davies, Quintanilha, Brooks, & Packer, 1982). Previously thought of as an unavoidable biproduct of exercise, ROS, and the resulting oxidative damage were originally thought to have a negative effect on health and the effectiveness of exercise. This led to a wide range of research into methodologies and supplementation regimes which aim to reduce the production of ROS during exercise and bolster the effectiveness of the multifaceted antioxidant defence system present within skeletal muscle. Antioxidant treatments, such as antioxidant loading of skeletal muscle, affect a wide range of signalling pathways both during and immediately following exercise (Merry & Ristow, 2016; S. K. Powers & Jackson, 2008). Studies investigated the effects of antioxidant loading on the adaptive response of skeletal muscle to exercise in order to help understand the way in which the reduction in oxidative stress affects muscular response.

High levels and long exposure to ROS within skeletal muscle is known to contribute to contractile dysfunction that occurs during fatigue (Reid, 2016). The low levels of ROS present within muscle at rest are required for normal force production, while a modest rise in ROS levels can increase muscle force production through an increase in Ca^{2+} transients and inhibiting Ca^{2+}-dependent ATPase (Reid, 2001b; Reid, Khawli, & Moody, 1993). At higher concentrations, these effects are reversed and ROS causes a decrease in contraction force and contributes to exercise-induced muscular fatigue (Reid, 2001a). This is brought about due to ROS causing decreased Ca^{2+} sensitivity in the muscle, inhibiting Ca^{2+} release and reuptake along with increased Ca^{2+} leak from the sarcoplasmic reticulum, and inhibiting the activity of metabolic enzymes. The knowledge of ROS involvement in fatigue led to research in which scavenging of ROS was upregulated in the hope to delay the onset of fatigue. Studies have shown that overexpression of antioxidant enzymes, as well as antioxidant treatments significantly delayed fatigued, allowing for greater contractile force over a longer period of time with effects being most noticeable during endurance exercise (Bruton et al., 2008; Davis, Murphy, Carmichael, & Davis, 2009; Novelli, Bracciotti, & Falsini, 1990).

Both at rest and during exercise, transient oxidative bursts allows ROS to act as signalling molecules. Redox regulation of proteins within muscle cells typically occurs through the reversible oxidation of cysteine thiols on the target, changing its activity, protein-protein interactions or cellular localisation (Holmstrom & Finkel, 2014). Many redox-sensitive signalling pathways are activated by ROS in muscle, including the AMPK, MAPK and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways, which control a range of metabolic and physiological adaptations (Ji, 2007). The activation of these pathways can lead to increases in muscle antioxidant capacity, protein synthesis and metabolic capacity. In this regard, exercise can have beneficial effects during immobilisation and aging and help prevent the onset of many pathological conditions such as diabetes and cancer by triggering these positive adaptations (Bajotto & Shimomura, 2006; Chopard, Hillock, & Jasmin, 2009; Ji & Yeo, 2019).

2.7 Antioxidant and Enzyme Interactions

2.7.1 AMPK S-glutathionylation

Studies have shown that the activity of AMPK is able to be modified by S-glutathionylation (Dong et al., 2016; Klaus et al., 2013). S-glutathionylation is a post-translational modification made to proteins which involves the formation of a mixed disulphide bond between an oxidised cysteine residue and glutathione (Corcoran & Cotter, 2013). Klaus et al. (2013) showed that several forms of mammalian glutathione S-transferases (GST) can interact with and activate AMPK through glutathionylation *in vitro*. This study focused on the activation of AMPK when the redox state of the cell was relatively low. Zmijewski et al. (2010) investigated changes in AMPK activity during more oxidative redox states and showed that higher concentrations of H_2O_2 were able to directly activate AMPK through oxidative modification of the AMPKα subunit. This is likely one of the many mechanisms and modifications, along with glutathionylation, that increases AMPK activity within muscle cells in response to increased levels of ROS following exercise.

The mammalian -Mu and -Pi classes of GSTs were further investigated and found not to interact with the α-subunit of AMPK (Klaus et al., 2013). Results showed that GST interacts with the very N-terminal part of the β-subunit of AMPK. The study concluded, using *in vitro* and *in vivo* models, that these GSTs interacted and bound with AMPK directly at the β-subunit, forming a complex which activates the enzymatic activity. This leads to the GST-facilitated S-glutathionylation of AMPK, activating signalling cascades within the cell in response to mildly oxidative intracellular conditions. The conclusions drawn from the Aranda et al. (2009) study are limited due to the yeast cell models and rat liver *in vivo* models it used, but the study also proposed a model of glutathionylation-dependent AMPK activation. Further studies are required to explore the importance of this mechanism in muscle, *in vivo*. Furthermore, the involvement of GSH and GSSG as a part of the S-glutathionylation reaction involved in the activation of AMPK needs to be explored.

Together, the interaction of GSTs with the α- and β-subunits of AMPK in HEK293 cells; the direct interaction of GST complexes with the β-subunit promoting S-glutathionylation; and kinase activity *in vitro*, provides strong evidence of the involvement of GSH in the regulation of muscle cell responses and adaptation to increased intracellular ROS by AMPK glutathionylation following exercise.

2.7.2 MEKK1 Glutathionylation

MAPK/ERK kinase kinase 1 (MEKK1) is an upstream activator of the stress-activated protein kinase (SAPK) signalling cascade (a member of the MAPK family) (Minden et al., 1994; M. Yan et al., 1994). MEKK1 is known to play a crucial role in the activation of many intracellular signalling pathways, including ERK, in response to extracellular stimuli. Research has shown that MEKK1 is redox sensitive, being inhibited through glutathionylation at a cysteine residue in its ATP-binding site (Cross & Templeton, 2004). This inhibition is suggested to be dominant over activation through the kinase phosphorylation cascade activated during exercise. This appears to allow for stimuli which cause oxidative stress in the cell to activate some pathways, while the increase in GSH, and therefore glutathionylation, inhibits others, as is the case with MEKK1.

2.8 Muscle Adaptations Following Exercise

2.8.1 Improved Fatty Acid Metabolism

A range of fatty acids are available and utilised as metabolic fuel by muscle during exercise. Initially intramyocellular triglycerides are the primary source. As the duration of exercise extends, the cell switches to ATP production from free fatty acids in plasma (Baker et al., 2010; Hargreaves & Spriet, 2020; Jeukendrup, 2002; Sidossis, Gastaldelli, Klein, & Wolfe, 1997). Other sources include very-low-density lipoprotein-triglycerides (VLDL-TG) which contribute around only 5% of total energy expenditure during exercise. The hydrolysis of VLDL-TGs is regulated by the activity of lipoprotein lipase (LPL). Skeletal muscle LPL, required to maintain energy balance and availability in the muscle, is known to be upregulated following exercise (Seip & Semenkovich, 1998). Strenuous or prolonged physical activity increases enzymatic activity, transcription, and translation of LPL, contributing to the restoration of energy balance by providing fatty acids as fuel while glycogen is depleted or being resynthesized (Kiens, 2006).

Various fatty acid transport proteins facilitate the uptake of free fatty acids. Transport of fatty acids into the mitochondria depends on the regulation of two carnitine palmitoyl transferases (CPT-1/CPT-2). CPT-1 enables the transport of long-chain fatty acids through the addition of carnitine. The fatty acid is then able to be transported by acyl carnitine transporters. This is the initiating and rate limiting step for oxidation of fatty acids within the mitochondria (Song et al., 2010). The activity of these transferases, specifically CPT-1, are upregulated during exercise as a result of upstream AMPK signalling. This, along with the availability of glycolytic products and free carnitine required for CTP-1 function, also increase the uptake, and consequently oxidation, of fatty acids into the mitochondria during exercise (Melanson, MacLean, & Hill, 2009).

Peroxisome proliferator activated receptor (PPAR) alpha is a transcription factor expressed in skeletal muscle. PPARα is lipid-activated and controls a range of genes involved in β-oxidation within the mitochondria (Minnich, Tian, Byan, & Bilder, 2001). Following exercise, PPARα expression is increased in muscle, this is facilitated by the activation of AMPK, directly leading to increased mRNA expression of PPARα target genes along with PGC-1α (Lee et al., 2006). This link between PPAR and PGC-1α expression shows one of many links between the increase of a cell's oxidative capacity and increased mitochondrial biogenesis. Endurance exercise is known to increase AMPK activity which leads to an acute increase fatty acid oxidation within the muscle. Along with these acute changes, the elevated AMPK activity causes long-term improvements in fatty acid metabolism due to altered gene expression including PPARα, PGC-1α and CPT-1.

Another isoform of PPAR, PPARδ, has also been found to increase the oxidative capacity of skeletal muscle following exercise. The expression of PPARδ is known to be increased in human skeletal muscle following endurance exercise. Muscle-specific overexpression studies have shown significant increases in fatty acid utilisation within the muscle (Luquet et al., 2003). Along with increased oxidative capabilities, other adaptive responses to exercise, such fibre type switching towards type 1 fibres, were also present (Y. X. Wang et al., 2004). This indicates that the PPARδ nuclear receptor therefore plays a central role in muscle adaptation following exercise and may be beneficial for the prevention of metabolic disorders such as diabetes and obesity (Lopez-Soriano, Chiellini, Maffei, Grimaldi, & Argiles, 2006; Luquet et al., 2003).

A third form of PPAR, PPARγ, is known to regulate lipid and glucose metabolism, helping protect skeletal muscle from excessive lipid overload (Phua, Wong, Liao, & Tan, 2018). Ligands for PPARγ such as polyunsaturated fatty acids, and many type 2 diabetes (T2D) medications, such as thiazolidinediones (TZDs), are known to improve glucose metabolism in muscle by activating PPARγ, leading to downstream PPARγ-mediated gene activation. The basal level of PPARγ activation maintains normal physiological insulin signalling, whereas in T2D patients the activity of PPARγ is significantly lowered (Janani & Ranjitha Kumari, 2015). Lack of PPARγ activity is linked to abnormal glucose metabolism and can lead to increased lipid storage within muscle. PPARγ modulates the transcription of a number of genes involved in glucose metabolism within muscle, most notably GLUT4. PPARγ activation leads to an increase in GLUT4 transcription and therefore increases insulin-stimulated glucose transport into the cell (Leonardini, Laviola, Perrini, Natalicchio, & Giorgino, 2009).

Calcium/Calmodulin Dependent Protein Kinase II (CaMKII) has been shown to regulate lipid metabolism in skeletal muscle in response to exercise (Mukwevho, 2017). Following exercise, increased CaMKII expression leads to beneficial changes, including increasing the expression of genes responsible for fatty acid metabolism in muscle (Joseph, Ayeleso, & Mukwevho, 2017). The accumulation of various lipids within the cell is associated with a wide range of pathological conditions including type 2 diabetes and obesity (Kinfe, Belay, Joseph, & Mukwevho, 2013). Increased CaMKII expression, in comparison to basal levels, has been shown to promote the breakdown of these lipids and restore a healthier level of lipids within cell membranes. CaMKII activation also leads to a decrease in the expression of acetyl-CoA carboxylase (ACC)-1, an enzyme that modulates the balance between lipid synthesis and oxidation within muscle. The combination of increased CPT-1 and decreased ACC-1 expression is known to decrease lipid accumulation and increase the oxidative capacity of muscle mitochondria. CaMKII activation resulting from exercise also stimulates mitochondrial biogenesis (details in section 2.8.6 Mitochondrial Biogenesis), which increases the mitochondrial density of muscle cells and activity of related enzymes. This is thought to increase the capacity of the cell to oxidise fat, also during exercise. Studies are yet to conclude if there is a direct correlation between mitochondrial density and lipid oxidation (Galgani et al., 2012).

2.8.2 Improved Glucose Metabolism

The glucose transporter GLUT4 is the most abundant GLUT protein in skeletal muscle. At rest a small portion of GLUT4 is located on the cell surface (~20%), while the remainder is stored in vesicles within the cell. Following stimulation by insulin or exercise, these vesicles translocate and fuse to the plasma membrane increasing the number of GLUT4 transporters therefore increasing the transport of glucose into the cell. The total levels of GLUT4 have been shown to increase between 20-70% in both human and rodent skeletal muscle following exercise.

The expression of GLUT4 within skeletal muscle is tightly regulated. Its upregulation following exercise means GLUT4 is involved in a wide range of adaptations to contraction. There are several signalling pathways involved in this upregulation. Two of the main signalling kinases known to regulate its expression are AMPK, and CaMKII. The activity of both enzymes increases during and following exercise. This response leads to the binding of the myocyte enhancer factor 2 (MEF2) family of transcription factors to the GLUT4 promoter and increased transcription. Several isoforms of MEF2 are expressed in skeletal muscle with MEF2A, MEF2C, and MEF2D being the most active. A heterodimer of these transcription factors is known to be involved in the normal regulation of GLUT4, binding to the GLUT4-MEF2 domain (Thai, Guruswamy, Cao, Pessin, & Olson, 1998).

The transcriptional coactivator PGC-1α also plays a role in the regulation of GLUT4 within skeletal muscle, including its response to exercise. PGC-1α directly binds to and coactivates MEF2C. Studies have shown that activation of MEF2C requires the coactivation of PGC-1α to enable control of the level of endogenous GLUT4 through binding to the promoter of the GLUT4 gene and inducing transcription (Michael et al., 2001). As previously stated, the expression and activity of PGC-1α is significantly increased following exercise, suggesting that its interaction with MEF2 may play a role, along with AMPK and CaMKII, in the increase in GLUT4 expression observed post-exercise. It is important to note that another downstream target gene of PGC-1α, NRF1, has also been suggested to affect the expression of GLUT4. Overexpression studies have also shown that NRF1 increased both GLUT4 expression and glucose transport in muscle (Baar et al., 2003).

The MEF2 binding site in the GLUT4 promoter is also subject to transcriptional repression by histone deacetylases (HDACs) 4, 5, and 7 (McKinsey, Zhang, & Olson, 2000). Under normal conditions HDAC5 is not a substrate for CaMKII, but upon dimerisation with HDAC4, it is capable of receiving signals from CaMKII (Backs, Backs, Bezprozvannaya, McKinsey, & Olson, 2008). The nuclear export of HDAC4/5, triggered by CaMKII and AMPK following exercise, allows for hyperacetylation of the GLUT4 promoter encouraging the binding of MEF2 (Mukwevho et al., 2008). This leads to an increase in GLUT4 expression further demonstrating the complex regulation of glucose metabolism in skeletal muscle.

HDAC5 is also known to be an AMPK substrate (Ojuka, Jones, Han, et al., 2002; Ojuka, Jones, Nolte, et al., 2002). The increase in AMPK activity during and following exercise acts in a similar way to CaMKII, working via HDAC4/5. Following exercise, active AMPK directly phosphorylates HDAC5, allowing for the acetylation of the MEF2 binding site in the GLUT4 promoter (Holmes, Sparling, Olson, Winder, & Dohm, 2005). Endurance exercise has been shown to increase the phosphorylation of AMPK and result in changes in gene expression favouring the translocation of GLUT4 in skeletal muscle (Rohling, Herder, Stemper, & Mussig, 2016). These changes improve the capacity for the muscle to effectively utilise glucose during exercise and shows an adaptation which extends past the acute effects of exercise on GLUT4.

Phosphofructokinase-1 (PFK-1) is a key enzyme involved in glucose breakdown within skeletal muscle, often termed as the pacemaker or rate-limiting step of glycolysis. PFK-1 catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate and requires ATP for this process (Lavorato, Andrade, Silva, Alves, & Oliveira, 2012). The breakdown of glucose is key for the rapid production of ATP during exercise when the muscle is under maximum contraction. PFK-1 activity has been shown to increase immediately following intense exercise as well as chronically following endurance exercise training (Abernethy, Thayer, & Taylor, 1990). These properties make PFK-1 a useful indicator of metabolic status within muscle following exercise.

Along with PFK-1, hexokinase (HK) plays a key role in the utilisation of glucose as an ATP source in skeletal muscle. HK catalyses the conversion of glucose to glucose-6-phosphate, transferring a phosphate group from ATP to the glucose molecule. HK activity is known to increase during and following exercise much like PFK-1 (Rockl et al., 2007). Skeletal muscle has been shown to maintain and/or increase HK expression following exercise (Lamb, Peter, Jeffress, & Wallace, 1969). Together, PFK-1 and HK are the key regulators of glycolysis and glucose utilisation within skeletal muscle. Alongside the increase in glucose transport via GLUT4, their elevated activity during exercise is crucial for the supply of ATP.

2.8.3 AMP-Dependent Regulation of AMPK

AMPK is one of the key regulators of skeletal muscle plasticity and energy metabolism, regulating the balance between anabolic and catabolic processes (Hardie & Carling, 1997; Jensen, Wojtaszewski, & Richter, 2009). AMP-dependent regulation is caused by the increase in AMP and ADP levels due to ATP hydrolysis, typically occurring during strenuous exercise. When cellular levels of AMP increase, it binds to the AMPK γ-subunit and allosterically increases the enzymatic activity of AMPK. This also induces phosphorylation of AMPK by liver kinase B1 (LKB1), further activating the enzyme (Long & Zierath, 2006). This leads to the downstream phosphorylation by AMPK of a wide range of target proteins, resulting in changes in metabolism and gene expression (Garcia & Shaw, 2017). Increased levels of AMPK activity following contraction was first documented by Winder and Hardie (1996). Many of the downstream targets of AMPK allow the cell to respond to increased energy requirement and there is now an extensive body of research surrounding AMPK activation and its role in maintaining energy homeostasis within cells (Carling et al., 2011; Hardie et al., 2011; Hardie et al., 2012). AMPK responds to metabolic stress, redirecting cellular metabolism towards increased catabolism and decreased anabolism resulting in reduced ATP consumption and increased ATP production (Herzig & Shaw, 2018). This is achieved in the short-term by phosphorylating rate-limiting enzymes while also having long-term effects by bringing about changes in gene expression (mentioned throughout 2.8 Muscle Adaptations following Exercise). During and immediately following contraction the AMPK pathway induces GLUT4 translocation resulting in increased glucose uptake. Furthermore, AMPK inhibits the synthesis of fatty acids, via ACC inhibition, while increasing their transportation to the mitochondria for ATP production through fatty acid oxidation (Long & Zierath, 2006). Alongside its effects on ATP production, AMPK also inhibits mTOR, reducing protein synthesis during and following periods of heightened ATP demand (Thomson, 2018).

2.8.4 AMP-Independent Regulation

Recent research has found that AMPK can also be activated independently of AMP/ADP concentration within the cell, known as AMP-independent regulation (Hardie, 2016; Xiao et al., 2013; Zhang et al., 2017). The first instances of AMPK activation without the direct effect of AMP were seen in a range of natural and synthetic small molecule activators. Molecules such as salicylate bind at what has become known as the Allosteric Drug and Metabolite (ADaM)-binding site of AMPK (Hawley et al., 2012). This is located at the interface of the α- and β-subunits of AMPK and is independent of the AMP binding site. When salicylate binds at this site it causes a conformational change in AMPK which transforms it into its active state, completely independently of cellular AMP levels.

Further research has suggested that ROS can also act as a form of AMP-independent regulation (Auciello, Ross, Ikematsu, & Hardie, 2014; Emerling et al., 2009; Shao et al., 2014). Zmijewski et al. (2010) showed that AMPK activation increased in response to exogenous H_2O_2 without any changes in AMP concentrations. Initially the mitochondria were suggested as one of the key locations for ROS production in muscle cells and therefore became a primary target for redox signalling studies (Chouchani et al., 2014). Although this was an attractive concept, similar studies were published which suggested that the only redox control, which AMPK was subject to, was due to the associated change in AMP levels within the cell (Auciello et al., 2014; Shao et al., 2014). This would suggest that redox signalling falls into the category of AMP-dependent regulation. Some of these contradictory points between studies are likely in part due to methodological differences including the cell types used, varying levels of ROS which the cells were exposed to, and differing methods of measuring adenine nucleotide levels. One of the more recent studies conducted by Hinchy et al. (2018), aimed to investigate whether AMPK was directly activated through interactions with ROS, or if the resulting intracellular changes in the ATP/AMP ratio indirectly lead to its activation. This study focused on ROS produced from the mitochondria along with exogenous H_2O_2 and used differentiated C2C12 mouse skeletal myotubes. Through simulated mitochondrial ROS production, they found no significant activation of AMPK. Although exogenous H_2O_2 stimulated AMPK activity, this was found not be the through direct redox signalling but was due to secondary changes in ATP/AMP ratios within the cell. The same study also explored these ideas using HEK 293T cells. In this model the exogenous H_2O_2 was found to activate AMPK independent of ATP/AMP ratios. These findings suggest that, along with indirect effects through changes in ATP/AMP ratios, there may be a direct way in which ROS stimulates AMPK signalling which is yet to be identified.

2.8.5 Mitochondrial Biogenesis

Exercise increases the volume, number and function of mitochondria within skeletal muscle (Leeuwenburgh & Heinecke, 2001; Safdar et al., 2011; Julie St-Pierre, 2003). This occurs through a process known as mitochondrial biogenesis. Muscle with a higher number of more efficient mitochondria has an increased oxidative capacity allowing for better performance as well as a range of protective health benefits (Hood, 2009).

PGC-1α was first identified as being involved in the differentiation of white fat to brown (Puigserver et al., 1998). The overexpression of PGC-1α in white fat gave the tissue some of the characteristics which are typically associated with brown fat such as a greater number of mitochondria and the stimulation of the expression of uncoupler proteins found in brown fat mitochondria. Since these initial findings it has been found that PGC-1α coactivates and binds to a wide range of transcription factors (Lindholm, Eriksson, Makela, Belluardo, & Korhonen, 2012).

PGC-1α is tightly regulated in skeletal muscle by a range of mechanisms both transcriptionally and post-transcriptionally, leading to the transcription of various genes involved in mitochondrial biogenesis. PGC-1α can be directly phosphorylated by p38 MAPK, AMPK, protein kinase A (PKA), Akt/PKB (Figure 3), or have its expression increased via cAMP-Response Element Binding Protein (CREB), and MEF2/NFAT (Figure 4) (Fernandez-Marcos & Auwerx, 2011).

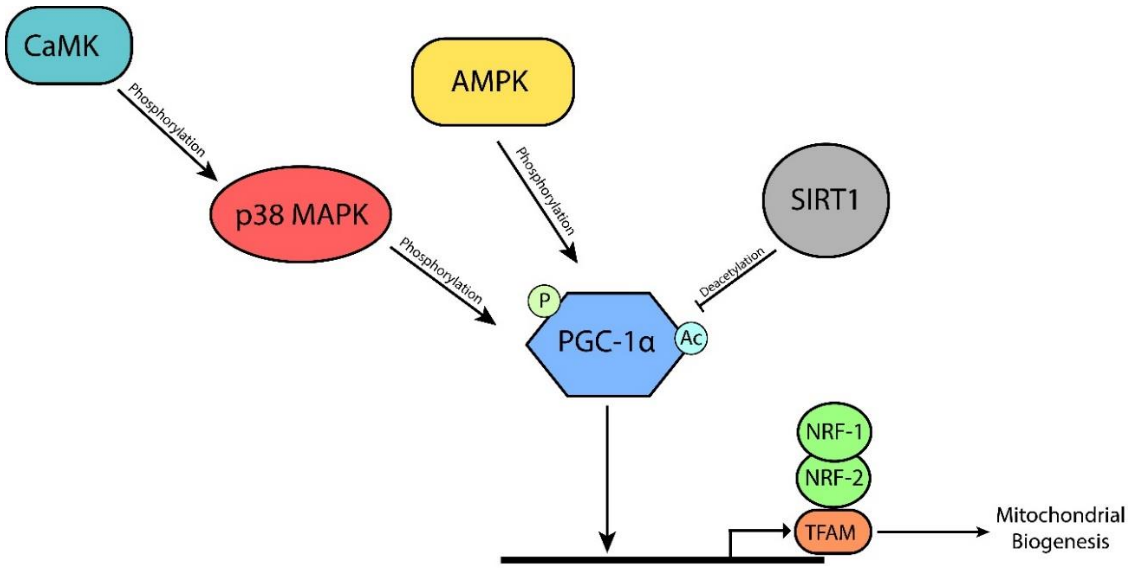

Figure 3. Control of mitochondrial biogenesis via PGC-1α. PGC-1α induces the expression of NRF1/2 and Tfam. These are the key genes which initiate mitochondrial biogenesis. PGC-1α also interacts with and co-activates NRF1, increasing its activity and enabling the maintenance of mitochondrial biogenesis in skeletal muscle. PGC-1α activity can be enhanced in multiple ways. CaMKs and AMPK are activated during exercise. In brief, CaMKs in skeletal muscle are phosphorylated by upstream CaMKK in response to increased sarcoplasmic calcium concentrations. This leads to the downstream phosphorylation of p38 MAPK. Both AMPK and p38 MAPK increase the stability of PGC-1α through phosphorylation. SIRT1 deacetylates PGC-1α effecting its localisation in the cell, enabling it to interact with a range of transcription factors within the nucleus, inducing the expression of genes including Tfam, NRF1 and 2.

The relatively short half-life of PGC-1α (two-three hours) is significantly increased upon phosphorylation by p38 MAPK and AMPK (Fernandez-Marcos & Auwerx, 2011; Jäger, Handschin, St-Pierre, & Spiegelman, 2007; D. Lindholm et al., 2012; Lira, Benton, Yan, & Bonen, 2010; Sano et al., 2007). This increased stability is crucial to allow PGC-1α to play its part in the regulation of mitochondrial biogenesis. The p38 MAPK elicits a dual action of regulation over PGC-1α by also blocking the interaction between PGC-1α and the co-repressor p160MBP (Fan et al., 2004). Displacement of the p160MBP co-repressor allows increased transactivation, further increasing the activity of PGC-1α.

Increased levels of phosphorylated (active) AMPK have been shown following contractile exercise (Takekoshi et al., 2006), first documented by Winder and Hardie (1996). This causes the downstream phosphorylation of a wide range of target proteins, including PGC-1α. Active AMPK directly phosphorylates the CREB transcription factor. The PGC-1α gene possess a binding site for CREB causing increased PGC-1α expression following the phosphorylation of CREB by AMPK (Fernandez-Marcos & Auwerx, 2011). Phosphorylation of PGC-1α by the active AMPK, increases the stability of the molecule, PGC-1α dependent activation of its own promoter is enhanced, and the protein interacts with other transcription factors such as MEF2 and PPARγ (Handschin & Spiegelman, 2006; Jäger et al., 2007). A similar increase in PGC-1α expression is seen following the phosphorylation of CREB by PKA. During exercise PKA is activated by the binding of cAMP to its regulatory subunits, causing these inhibitory subunits to dissociate and therefore activating the catalytic subunits of the enzyme (Taylor et al., 2004). This activation leads to the subsequent phosphorylation of CREB and an increase in PGC-1α expression within the cell.

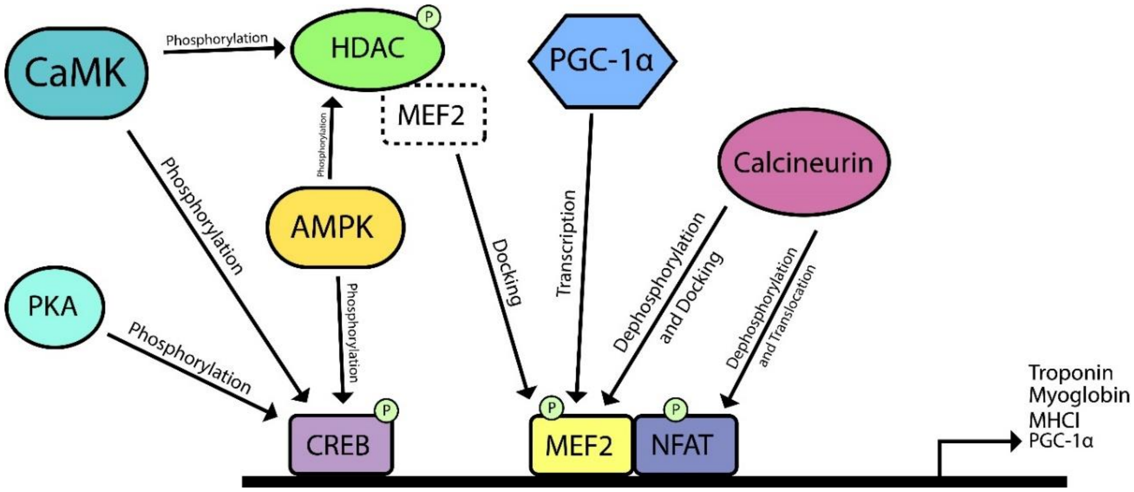

Figure 4. The expression of PGC-1α is regulated in various ways at the transcriptional level. PKA, AMPK and CaMK are activated in muscle following exercise and activate the CREB transcription factor by phosphorylation. When phosphorylated by AMPK and CaMK, histone deacetylases (HDAC) dissociate from MEF2 allowing it to bind to the PGC-1α gene and initiate transcription. PGC-1α causes an increase of its own transcription creating a positive feedback loop following exercise through the upregulation of MEF2 expression. Calcineurin is a Ca^{2+}/calmodulin dependent protein phosphatase and can directly dephosphorylate MEF2 and NFAT, inducing translocation of NFAT to the nucleus and binding of NFAT and MEF2 to the PGC-1α promoter inducing its transcription along with other genes, such as troponin, myoglobin and MHCI.

Along with phosphorylation by AMPK, PGC-1α can also undergo acetylation. This is carried out by the histone acetyl transferase, general control of amino acid synthesis 5 (GCN5) (Dominy, Lee, Gerhart-Hines, & Puigserver, 2010). The acetylation of PGC-1α causes it to localise from the promotor regions to the nuclear foci inhibiting its transcriptional activity (Lerin et al., 2006; Tang, 2016). One of the primary ways in which the total activity of PGC-1α is modulated within skeletal muscle is the balance between the acetylation activity of GCN5 and the deacetylation activity of sirtuin 1 (SIRT1). The protein deacetylase SIRT1 can be activated by AMPK, directly interacts with, deacetylates and activates PGC-1α, changing its localisation within the nucleus, allowing it to interact with and initiate the transcription of target genes involved in mitochondrial biogenesis (Cantó et al., 2009; Li et al., 2011).

Calcium is one of the most important second messengers in skeletal muscle, triggering muscle contraction as well as a range of signal transduction pathways including those leading to mitochondrial biogenesis (Pette & Staron, 1990). Intracellular levels of calcium correlate directly to contractile activity, and this allows Ca^{2+} to modulate the activity of downstream signalling pathways in proportion to contraction. Calmodulin is an intermediate calcium-binding messenger protein which undergoes conformational changes in response to changes in intracellular calcium concentrations (Stevens, 1983). Following these conformational changes, calmodulin activates a range of downstream signalling proteins. These include phosphatases such as calcineurin, as well as a number of CaMKs, which play a key role in the increasing the number and function of mitochondria in skeletal muscle following exercise (Swindells & Ikura, 1996).

CaMKII pathways are involved in the upregulation of mitochondrial biogenesis following exercise. CaMKII is phosphorylated in response to increases in cytosolic calcium and calmodulin concentrations (Jain et al., 2014). Such activity has been shown in studies where muscle placed under metabolic stress through the uncoupling of SERCA pump activity showed direct increases in CaMKII, recruitment of PGC-1α and stimulation of mitochondrial biogenesis (Maurya et al., 2018). CaMKII is one of the primary enzymes responsible for the phosphorylation of CREB and HDAC. CaMKII directly phosphorylates CREB, leading to the induction of PGC-1α along with other genes involved in the upregulation of mitochondrial biogenesis. Furthermore, CaMKII also phosphorylates HDAC. The histone deacetylase inhibits MEF2 activity when unphosphorylated, repressing PGC-1α transcription. When phosphorylated by CaMKII, HDAC dissociates from MEF2, allowing MEF2 to bind to the PGC-1α gene and initiate transcription (Lira et al., 2010).

CaMKIV has been shown to increase mitochondrial biogenesis in skeletal muscle as well as promoting a low level of fast to slow fibre type transition (Wu et al., 2002). Mitochondrial biogenesis is upregulated through the enhanced expression of PGC-1 by CaMKIV, via the phosphorylation of MAPK. This was shown in a study via the *in vitro* and *in vivo* upregulation of CaMKIV in skeletal muscle (Wu et al., 2002). This demonstrates another pathway through which calcium signalling is involved in the adaptive response of skeletal muscle through the expression of PGC-1α. Although CaMKIV is not highly expressed in skeletal muscle, other isoforms including CaMKII may have similar effects on the upregulation of target genes in muscle (Rose, Kiens, & Richter, 2006). This suggests that although CaMKIV can play a role in the physiological responses to exercise and is involved in muscle plasticity, it is not essential, with phenotypic changes able to take place without an increase in CaMKIV expression.

Calcineurin is a serine/threonine phosphatase activated in skeletal muscle by elevations in intracellular calcium levels during exercise (Sakuma & Yamaguchi, 2010). The downstream effects of activated calcineurin includes the expression of various genes involved in cell growth, maintenance, remodelling and mitochondrial biogenesis (Chin, 2004; Chin et al., 1998; Ryder, Bassel-Duby, Olson, & Zierath, 2003; Schulz & Yutzey, 2004). Overexpression of calcineurin in skeletal muscle has been shown to increase the expression of PGC-1α along with increasing the activity of mitochondrial enzymes (Naya et al., 2000; Ryder et al., 2003). Studies have also found that animals treated with a calcineurin inhibitor, Cyclosporin A, still maintain regular increases in PGC-1α expression following exercise, suggesting that calcineurin is not necessary for PGC-1α expression. Calcineurin affects PGC-1α expression by dephosphorylating nuclear factor of activated T cells (NFAT) c1. NFAT c1 is a transcription factor which was first characterised in the immune system with its activation requiring nuclear translocation, controlled by calcineurin. (Flanagan, Corthésy, Bram, & Crabtree, 1991; Schulz & Yutzey, 2004). Once dephosphorylated, NFAT is able to bind to the promoter region and induce transcription of PGC-1α (Jung & Kim, 2014). Furthermore, studies have shown that calcineurin is involved in mediating mitochondrial biogenesis following exercise via the activation of MEF2, another potent inducer of PGC-1α expression (Handschin, Rhee, Lin, Tarr, & Spiegelman, 2003; Wu et al., 2001b). MEF2 is also coactivated by PGC-1α, creating a positive feedback loop upregulating its own expression (Handschin et al., 2003).

NRF 1 and 2 are transcription factors directly upregulated by PGC-1α (Mootha et al., 2004; Wu et al., 1999). Following exercise, PGC-1α is upregulated and binds to and coactivates NRF1, significantly increasing its transcriptional activity on downstream target genes. These include the mitochondrial transcription factor A (Tfam). Once produced, Tfam enters the mitochondria where it directly stimulates mitochondrial DNA (mtDNA) replication and mitochondrial gene expression (Tang, 2016; Z. Wu et al., 1999).

The tumour suppressor protein p53 is involved in the rapid response to exercise within skeletal muscle (Beyfuss, Erlich, Triolo, & Hood, 2018). Its general function is to maintain cellular homeostasis during these periods of physiological stress. During exercise, p53 is activated by AMPK and p38 MAPK, which phosphorylate specific residues on p53 and increase the protein's stability. Once p53 is phosphorylated its mitochondrial and nuclear localisation increases. In mitochondria, p53 interacts with Tfam, causing induction of mitochondrial genes involved in mitochondrial biogenesis (Saleem, Carter, Iqbal, & Hood, 2011; Yoshida et al., 2003). In the nucleus p53 induces the expression of PGC-1α, contributing to increased mitochondrial biogenesis in response to exercise (Park et al., 2009).

Citrate synthase (CS) is a key enzyme involved in the first step of the Krebs cycle which is coded for by nuclear DNA and then transported into the mitochondrial matrix after synthesis. Following endurance exercise CS expression is increased in skeletal muscle and it is commonly used as a metabolic marker of mitochondrial density and oxidative capacity (Siu, Donley, Bryner, & Alway, 2003). This increase is likely due to increased CS synthesis rather than decreased degradation following exercise, with oxidative capacity of a muscle increasing in proportion to increases in CS activity (Vigelso, Andersen, & Dela, 2014). Long-term increases in muscle CS levels (e.g. following an 8-week endurance protocol) suggest that CS plays an important role in muscle adaptations to exercise allowing for increase oxidative capacity.

2.8.5.1 The Effects of Antioxidants on Mitochondrial Biogenesis

One of the first studies directly linking antioxidant supplementation with a decrease in mitochondrial biogenesis in response to exercise when compared to controls was published in 2008 (Gomez-Cabrera et al., 2008). This study was later supported by Strobel et al. (2011) who showed that daily antioxidant supplementation decreased markers for mitochondrial biogenesis, including PGC-1α and NRF1, and mitochondrial enzyme activity following treadmill training in rats. In the following years, further studies were published on this topic exploring the link between antioxidants and their effect on mitochondrial adaptation to exercise. After more in depth investigation, it was found that although antioxidants do decrease the markers of mitochondrial biogenesis following exercise this did not translate into a decrease in mitochondrial mass, which still increased compared to controls following exercise (Abadi et al., 2013; Meier et al., 2013). Results from targeted antioxidant treatments as well as general antioxidant loading experiments suggest that the effect of antioxidants on muscle mitochondrial responses to exercise was dependent, not only on the method and duration of antioxidant supplementation but also the volume of training (Higashida, Kim, Higuchi, Holloszy, & Han, 2011; Merry & Ristow, 2016).

Evidence from animal models suggest that in many cases antioxidant treatment decreases the markers of mitochondrial biogenesis, such as PGC-1α, in skeletal muscle in response to exercise. This finding was supported in human trials. Paulsen et al. (2014) found that increases in PGC-1α mRNA seen following endurance training were prevented by supplementation with vitamins C and E (Padayatty et al., 2003; X. Wang & Quinn, 1999). However, a study by Yfanti et al. (2010) came to opposing conclusions, finding that the administration of vitamins C and E had no effect on the physical adaptations to exercise. Although this study did not find differences between control groups and those treated with antioxidants, it did not directly measure markers of mitochondrial biogenesis but instead measured CS activity. This limits the ability to draw conclusion from this study as to the effects within Krebs cycle activity, which while although linked to mitochondrial volume, it may not accurately reflect muscle mitochondria number and activity (Siu et al., 2003; Srere, 1969)

This potentially conflicting data surrounding the effects of antioxidants on mitochondrial biogenesis in response to exercise suggests that, although antioxidants are capable of supressing this response in skeletal muscle, there are most likely other mechanisms at work along with redox signalling which allow for a normal physiological response. Until more recently, studies were conducted with global antioxidants which scavenge free radicals in a non-specific manner. These include vitamins E and C which were widely used when looking at the effects of antioxidants in skeletal muscle adaptation to exercise. These studies provided a solid foundation of knowledge on the potential effects of antioxidants on signalling pathways involved in adaptation of skeletal muscle. Further research utilising targeted site-specific antioxidants is required in order to understand the specific effects and changes caused by antioxidants in skeletal muscle due to the highly compartmentalised nature of redox signalling within muscle.

2.8.6 Fibre Type Switch (glycolytic to oxidative)

Skeletal muscle is highly plastic and exercise is known to induce changes in fibre type composition (Yan, Okutsu, Akhtar, & Lira, 2010). Fibre type composition of skeletal muscle has been well researched as it directly affects both physical performance and the incidence of disease (Hickey et al., 1995; Inbar, Kaiser, & Tesch, 1981). Changes in the expression of a wide variety of genes are involved in the transition of muscle fibres from one type to another and many of these are influenced by exercise (Chin et al., 1998). Endurance exercise promotes an increase in the proportion of type I slow-twitch oxidative muscle fibres and type II fast-twitch oxidative fibres (type IIa). These are mitochondria dense and use oxidative production of ATP to supply a steady energy source during long periods of aerobic exercise (Wang et al., 2004).

The role which CaMK plays in muscle remodelling has been widely researched as it is suggested to activate MEF2 and NFAT regulated genes by alleviating the repression of HDACs (McKinsey et al., 2000). MEF2 is a transcription factor which is highly expressed in skeletal muscle and neurons while also being expressed at lower levels in a wide range of other cell types (Black & Olson, 1998). In skeletal muscle, MEF2 transcription factor binds to the promoter regions of genes involved in proliferation and differentiation, such as the MyoD family, along with genes controlling the formation of slow-twitch oxidative muscle fibres, which includes PGC-1α. Following endurance exercise, MEF2 expression has been shown to increase as the muscle adapts to the prolonged contraction resulting in a higher percentage of slow-twitch fibres in skeletal muscle (Wu et al., 2001a).

PGC-1α is suggested to be involved in the transition of muscle fibres from type II to type I (Handschin et al., 2007). Endurance exercise and overexpression studies of the protein have shown that it is directly involved in the increase of type I muscle fibres (Calvo et al., 2008; J. Lin et al., 2002). Although evidence showing the involvement of PGC-1α in the promotion of mitochondrial biogenesis is substantial, both it and the evidence surrounding the role in fibre-type switching has been challenged (Gouspillou et al., 2014; Rowe, El-Khoury, Patten, Rustin, & Arany, 2012). These challenges arise from potential disparities between PGC-1α function, in regard to fibre specific function, in human and rodent models. Variance between fibre type-specific PGC-1α regulated genes in humans and rodents along with a study which shows that PGC-1α may not be required for metabolic remodelling of skeletal muscle provoke questions around the transcriptional coactivator’s role in adaptation and fibre type switching (Gouspillou et al., 2014; Pérez-

Schindler, Summermatter, Santos, Zorzato, & Handschin, 2013). Another study showed that PGC-1α is dispensable for exercise-induced mitochondrial biogenesis in skeletal muscle, rather than only being required for developmental mitochondrial biogenesis and for the response to acute physiological stress (Gan, Fu, Kelly, & Vega, 2018; Rowe et al., 2012).

Rockl et al. (2007) and Yan et al. (2010) showed that AMPK signalling is required for muscle fibre type switch following exercise. The study of muscle tissue in mice following an endurance exercise protocol showed that muscle type transition from IIb to IIa/x was significantly decreased in transgenic mice with an inactive AMPKα2 subunit compared to wild type mice. The shift in fibre type towards more oxidative types of muscle fibre was not completely blunted by the inactive AMPKα2 subunit suggesting that AMPK is not solely responsible for this muscle adaptation. The significant difference in these two groups provides strong evidence that AMPK is directly involved in and required for normal muscle fibre type adaptation following contraction.

Levels of PPARβ/δ increase in muscle following repeated bouts of exercise and overexpression of PPARβ/δ leads to an increase in the percentage of type I fibres, whilst its deletion caused a decrease in type I muscle fibre gene expression (Schuler et al., 2006; Spangenburg, Brown, Johnson, & Moore, 2009; Wang et al., 2004). Although these observations clearly suggest the involvement of PPARβ/δ in the transition of muscle fibres from type II to type I, it is yet to be determined if the switch is dependent on the transcriptional activity of PPARβ/δ (Yan et al., 2010).

The study of exercise-induced muscle fibre type transition can be heavily influenced by the methodology used in each individual study. Myofibrillar myosin ATPase histochemistry is commonly used to determine muscle fibre composition, and is significantly different from various antibody based analyses which are also commonly used in similar studies with these two methods potentially producing differing results (Chin et al., 1998; Naya et al., 2000; Pogozelski et al., 2009; Wang et al., 2004). Differing conclusions have been drawn from similar studies, warranting caution in the interpretation of results (Geng et al., 2009; Potthoff et al., 2007). Nevertheless, physiological models appear to be the most reliable sources in the search for the regulators of fibre type switching in skeletal muscle at the current time.

ERK1/2 is a MAPK involved in the switch from type II to type I fibres in skeletal muscle. ERK1/2 have been found to be redox sensitive, with levels of ROS at rest, as well as following exercise, adequate for activation of the enzyme and thus increased activation seen following exercise (Kefaloyianni, Gaitanaki, & Beis, 2006). Animal models have suggested that the activation of ERK2 but not ERK1 is required for the switch to a more oxidative and fatigue-resistant fibre type, although this may be due to the relative abundance of the two ERK isoforms in skeletal muscle (Boyer et al., 2019). The mechanism of ERK1/2 activation is through an upstream GTPase called Ras. Activation of Ras in the plasma membrane leads to the recruitment and phosphorylation of Raf-1 kinase (a MAPKKK), which phosphorylates and activates MEK1/2 (a MAPKK) which then phosphorylates and activates ERK1/2 (a MAPK) (Mandal, Becker, & Strebhardt, 2016; Migliaccio et al., 2013). Early studies suggested that ROS was required for the activation of ERK1/2 in skeletal muscle (Gomez-Cabrera et al., 2008). The reduction in ROS, specifically reactive oxygen and nitrogen species (RONS), produced following XO inhibition, blunted ERK1/2 activation, suggesting that the ROS produced by XO play a key role in the activation of ERK1/2 signalling pathways. Subsequent studies showed the activation of ERK1/2 by ROS at resting concentrations (Kefaloyianni et al., 2006). More recently, work with animal models has shown that ERK1/2 signalling is directly responsible for the switch towards type II slow fibre types *in vivo* (Boyer et al., 2019). In cardiac muscle it has already been established that ERK1/2 is involved at multiple levels alongside MEK1 and calcineurin/NFAT signalling pathways (Sanna, Bueno, Dai, Wilkins, & Molkentin, 2005).

Hypoxia-inducible factor 2 (HIF-2) is a transcription factor, closely related to HIF-1, and present in skeletal muscle, which controls many aspects of the cell’s response to hypoxia. HIF-2 may be involved in long-term adaptations to hypoxia rather than acute changes in gene expression seen during exercise (Lendahl, Lee, Yang, & Poellinger, 2009). Importantly, HIF-2 expression increases following exercise and increases the expression of a wide variety of genes associated with slow-twitch oxidative muscle fibres such as myosin heavy chain I (MHCI), myoglobin and calmodulin2 (Lindholm & Rundqvist, 2016; Rasbach et al., 2010). This induction of genes by HIF-2 is also required for PGC-1α induced fibre type switching. PGC-1α specifically increases the expression of HIF-2 and is involved in a wide range of adaptations to exercise including mitochondrial biogenesis.

The process of fibre type switching following exercise is complex and required a wide range of molecular adaptations to take place. These type I fibres are referred to as being slow twitch oxidative fibres and are characterised by having higher mitochondrial content and increased capillary density when compared to more glycolytic fibre types (Wen et al., 2014). PGC-1α plays a key role in the switching of fibres towards a more oxidative type I composition following endurance exercise. One of the primary characteristics of oxidative fibres is greater expression of MHCI which when compared to MHCII. The MHCI isoform of the motor protein has a lower ATP consumption rate, due to lower ATPase activity, and produces slower contractions (Han, Geiger, Cody, Macken, & Sieck, 2003; He, Bottinelli, Pellegrino, Ferenczi, & Reggiani, 2000). Increased PGC-1α expression along with MEF2 and NFAT activation, such as that seen following exercise, induces the transcription of MHCI and of multiple other genes, including myoglobin and troponin (Lin et al., 2002). The expression of MEF2, induced by PGC-1α activity, feedbacks to further increase PGC-1α expression, leading to greater increases in regulated genes. Higher levels of myoglobin are a key characteristic of oxidative type I fibres. Myoglobin levels are known to increase following endurance training and this is once again due to the increase in expression of PGC-1α which induces myoglobin expression (Lin et al., 2002; Ordway & Garry, 2004). These increased myoglobin levels within the muscle favour greater oxygen delivery to the tissue (Gros, Wittenberg, & Jue, 2010). Additionally, increased PGC-1α expression results in oxidative and angiogenic improvement (Figure 5), complementing these adaptations and the transition towards a more oxidative fibre type.

Type I fibres have higher mitochondrial density than other fibre types, meaning that the induction of mitochondrial biogenesis is key to the transition from glycolytic to more oxidative fibre compositions within the muscle. As previously described, the upregulation of PGC-1α following endurance exercise promotes the expression of genes involved in mitochondrial biogenesis. A greater mitochondrial density directly increases the oxidative capacity of the fibre contributing to the fatigue resistance of the muscle. Enzymes involved in ATP production through the Kerbs cycle and β-oxidation are also upregulated in type I fibres. PPARδ, a key regulator of fatty acid transport and oxidation, expression is markedly increased in type I fibres (Janani & Ranjitha Kumari, 2015; Wang et al., 2004). As lipid oxidation for ATP synthesis increases as exercise duration extends, PPARδ activity along with increases in fatty acid transporters, allows for greater endurance in exercise (Bradley et al., 2012).

Along with myoglobin, vascular endothelial growth factor (VEGF) aids in enhancing oxygen delivery required in type I muscle fibres. VEGF directly increases the oxygen availability to the muscle by promoting the development of new blood vessels, increasing total blood flow (Wagner, 2011). PGC-1α is a powerful regulator of VEGF, increasing its expression in both cultured muscle cells an *in* vivo (Arany et al., 2008). The control of VEGF by PGC-1α provides another layer to the involvement of PGC-1α in fibre type switching towards type I fibres following endurance exercise.

A wide range of factors contribute to the switching of skeletal muscle fibre type towards more oxidative fibre type compositions. The lower ATP consumption rate by MHCI and enhanced oxidative capacity through mitochondrial biogenesis increases fatigue resistance during endurance exercise. The MHCI isoform generates a lower mechanical power output but is more efficient in its utilisation of ATP in comparison to other isoforms found in type II fibres (Guth & Samaha, 1969; Leonardini et al., 2009; Mukund & Subramaniam, 2020; Rivero, Talmadge, & Edgerton, 1998). Type I fibres also have higher levels of antioxidant enzyme activity, such as SOD1, allowing ROS to be controlled over longer periods such as during endurance exercise (Criswell et al., 1993; Powers et al., 1994; Powers et al., 2011). The elevated expression of metabolic enzymes such as those involved in the Krebs cycle and β-oxidation, along with higher oxygen availability due to greater levels of myoglobin as well as VEGF regulated angiogenesis, facilitate extended periods of consistent aerobic contraction. The increased activity of PGC-1α following endurance exercise triggers many of these metabolic adaptations (Figure 5), contributing towards the switching to more oxidative and fatigue resistant fibre types.

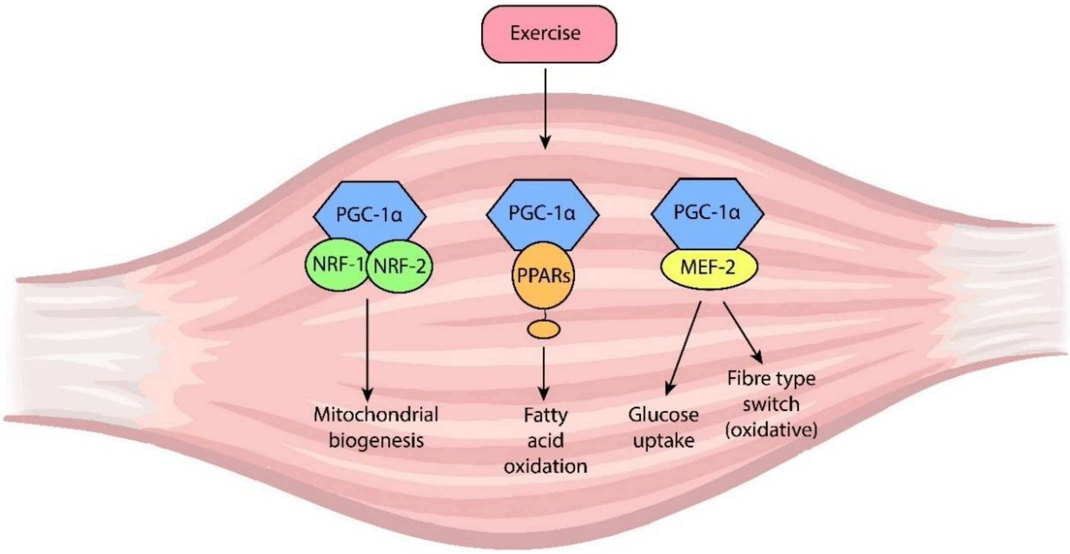

Figure 5. PGC-1α is a key regulator of skeletal muscle adaptation. Its expression and activity are both increased in following exercise. This can trigger a wide range of physiological and metabolic adaptations depending on the transcription factors with which PGC-1α interacts. The activation of NRF1 and 2 causes an increase in mtDNA replication and results in mitochondrial biogenesis. PPAR transcription factors activated by PGC-1α increase the muscle's oxidative capacity. PGC-1α can also increase the activity of the MEF2 transcription factor which has downstream effects in both the uptake of glucose into muscle through increased GLUT4 expression, as well as promoting a fibre type switch towards type I oxidative fibres

2.8.7 Angiogenesis

Angiogenesis, the growth of new blood vessels, is an important adaptation of skeletal muscle. Following exercise, upregulated expression of VEGF plays a key role in increasing the capillary/fibre ratio within muscle. The VEGF-induced capillary formation directly leads to an increase in exercise capacity (Olfert, Howlett, Wagner, & Breen, 2010), allowing for a greater amount of blood flow to muscle, improved delivery of nutrients and oxygen, as well as clearance of metabolites, increasing performance in later bouts of exercise. Conversely, a decrease in VEGF expression, such as during extended periods of inactivity, leads to reduced capillarity (Wagner, 2011).

VEGF is an important target for various clinical applications such as cancer treatment (Cardones & Banez, 2006). The idea of targeted VEGF reduction within tumour cells and therefore restricting blood flow is well researched yet there are still many complications and side-effects associated with current treatments. Along with PGC-1α, mentioned in the above section, the interactions of ROS and antioxidant systems have been found to affect VEGF expression. This opens a new area of investigation surrounding VEGF-mediated redox sensitive molecular processes (Neves et al., 2018).

HIF-1 is a transcription factor found is most human cells including skeletal muscle that acts as a master regulator controlling genes involved in the cellular response to hypoxia (Hoppeler, Vogt, Weibel, & Fluck, 2003). The two subunits, HIF-1α and HIF-1β/ARNT are present in both the nucleus and cytosol and are able to shuttle between the two (Gassmann, Chilov, & Wenger, 2002). HIF-1 levels are primarily regulated through the adjustment of the rate of protein degradation of the HIF-1α subunit (M. E. Lindholm & Rundqvist, 2016). At rest, when the cell is in a state of normoxia, HIF-1α is hydroxylated by HIF-prolyl hydroxylase domain-containing enzymes (PHD). The now present oxygen-dependent degradation domain (ODDD) is targeted by Von Hippel-Lindau E3 ubiquitin-ligases (VHL) for degradation through the ubiquitin-proteasome pathway (Zhu & Bunn, 1999). During periods of hypoxia, such as those induced by both acute and endurance exercise, the lack of oxygen no longer activates the ODDD and HIF-1α is not hydroxylated. The now stable HIF-1 accumulates within the nucleus of the cell (Ameln et al., 2005; Jewell et al., 2001). This then allows HIF-1α to dimerise with the HIF-1β/ARNT subunit also present within the nucleus. The HIF-1 dimer is then able to bind to DNA and induce the expression of a range of genes involved in the muscle's response to hypoxia such as VEGF (Ameln et al., 2005; Gassmann et al., 2002)

HIF-1α is stabilised by MAPKs under hypoxic conditions. This process requires mitochondria-derived ROS, specifically H_2O_2, in order to take place (Gassmann et al., 2002; Zhu & Bunn, 1999). Overexpression of catalase, which catalyses the dismutation of H_2O_2 into H_2O and O_2, inhibits the stabilisation of HIF-1α by MAPK (Emerling et al., 2005; Lindholm & Rundqvist, 2016). This along with other studies, show that MAPK activation, HIF-1α accumulation and the expression of VEGF are controlled by and require ROS generated within the mitochondria during exercise (Emerling et al., 2005; Emerling et al., 2009; Levy, Levy, Wegner, & Goldberg, 1995).

2.8.8 Improved Antioxidant Potential

The antioxidant activity and capacity of body tissues is closely related to the oxygen consumption and ROS production of that tissue. For example, brain tissue, which has some of the highest oxygen consumption rates, has one of the highest levels of antioxidant enzyme activity of any tissues. This is also true for skeletal muscles, with some having a higher antioxidant enzyme activity than others. Long-term or chronic exposure of skeletal muscle to higher levels of oxidants triggers an increase in expression and activity of antioxidant systems, counteracting the higher ROS levels in the cell (Oberley, St Clair, Autor, & Oberley, 1987). Muscle GSH content, along with overall antioxidant enzyme activity, is known to increase following regular endurance exercise (Ji, Leeuwenburgh, & Powers, 1999; Leeuwenburgh et al., 1997). The degree to which this level is increased is suggested to be muscle fibre type specific.

The oxidant/antioxidant balance which is maintained in muscle cells is perturbed during strenuous exercise when ROS production is increased, triggering an upregulation of antioxidants by the cell. This acute response has also been shown to cause a longer-term adaptation increasing the antioxidant potential of the muscle. As well as short bouts of exercise, endurance exercise also causes long-term changes in antioxidant enzyme levels including basal levels at rest (Higuchi, Cartier, Chen, & Holloszy, 1985; Quintanilha & Packer, 1983). Similar adaptations have been observed in other tissues, such as heart and liver, suggesting that the mechanism responsible for the long-term increase in antioxidant potential is not tissue specific (Kanter, Hamlin, Unverferth, Davis, & Merola, 1985; Sen, Marin, Kretzschmar, & Hanninen, 1992).

It has been shown that the activity of SOD, catalase, and GPx in skeletal muscle increase following exercise (Berzosa et al., 2011; Powers, Ji, & Leeuwenburgh, 1999). The degree to which these increases occur depends on the intensity and duration of the exercise. High-intensity exercise increased GPx activity more so than low-to-moderate activity. Furthermore, longer duration exercise led to greater increases in GPx activity in comparison to short-duration exercise. Along with GPx, SOD has been shown to increase following long-duration exercise. A study by Ji, Gomez-Cabrera, and Vina (2007) suggests that ROS produced during and following exercise is involved in the activation of NF-κB and MAPK pathways and that these are likely to be involved in the increased activity of antioxidant enzymes including SOD and GPx. Catalase has also been shown to significantly increase following both high intensity and lower continuous exercise in comparison to levels at rest, likely in response to elevated ROS levels (Berzosa et al., 2011).

The activity of extracellular GPx and SOD is also known to rise following exercise. This increase in activity was measured directly following an acute bout of exercise before they returned to basal levels one hour later. Interestingly, enzymatic activity increased again 24 hours post-exercise. This is suggested to be due to possible genetic upregulation of GPx and SOD (Berzosa et al., 2011; Elosua et al., 2003).

2.9 Conclusion

The generation of ROS is closely associated with intracellular signalling, both at rest and during exercise, with a range of antioxidant enzymes and molecules, such as GSH, involved in the maintenance of the cellular oxidant-antioxidant balance. (Powers et al., 2011). Chronic elevation of ROS and oxidative damage to proteins, DNA, and cell membranes can contribute to complications of a range of pathologies, including diabetes and cancer. On the other hand, at rest and during acute bursts, such as during exercise (Davies et al., 1982), ROS are maintained at a level where they act as important signalling molecules while causing minimal or reversible oxidative damage. ROS themselves are involved in signalling pathways which lead to the improvement of antioxidant potential, mitochondrial biogenesis, and fibre type switch. Antioxidants work to reduce this oxidative damage though the maintenance of the oxidant/antioxidant balance but, under acute or short-term oxidative stress, antioxidant molecules also play an active role in signalling. One example is GSH, which can cause the glutathionylation of important enzymes controlling muscle adaptations in response to exercise (Thirupathi & Pinho, 2018), but the extent to which GSH is involved in this adaptation is poorly understood. Therefore, the study aims to provide further understanding on the involvement of endogenous GSH in the molecular adaptation of skeletal muscle to contractions induced by ES.

3. Materials and Methods

3.1 Reagents

High glucose Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco by Life Technologies (MA, USA) (ref: 11995-065) along with 2.5% Trypsin (10x) (ref: 15090-046) and penicillin-streptomycin (ref: 15140-122). Horse serum (ref: 16050-122) was bought from Gibco by Life Technologies. Fetal Bovine Serum (FBS) (ref: MG-FBS0820) was purchased from Moregate Biotech (New Zealand). The Isolate II RNA Mini Kit (ref: BIO-52073), High Capacity cDNA Reverse Transcription Kit (ref: 4368813) and PowerUp SYBR Green Master Mix (ref: A25742) were obtained from Applied Biosystems by Thermo Fisher Scientific (MA, USA). Trypan Blue solution (ref: 93595) was obtained from Fluka Analytical. Glutathione Fluorescent Detection Kit (ref: EIAGSHF) and Amplex UltraRed reagent (ref: A36006) were purchased from Invitrogen by Thermo Fisher Scientific (MA, USA). Catalase from bovine liver (ref: C1345), L-

Buthionine sulfoximine (ref: B2515), Type II Horseradish Peroxidase (ref: P8250) and Aqueous 5-dihydrate Sulfosalicylic acid (SSA) (ref: S2130) were purchased from Sigma-Aldrich (Merck) (MO, USA). Primers were purchased from IDT (IA, USA).

3.2 C2C12 Cell Culture

C2C12 mouse myoblasts were maintained at 37 ^{o}C and 5% CO_2, in high glucose DMEM, 10% (FBS), and 2% Penicillin-Streptomycin, referred to as proliferation medium. Cells were routinely cultured in 75 cm^2 flasks prior to trypsinisation and seeding onto 6-well plates at a density of ~75,000 cells per well for stimulation. When these wells reached approximately 60-70% confluency, after between two and three days of incubation, the medium was changed to differentiation medium of high glucose DMEM with 2% Horse Serum and 2% Penicillin-Streptomycin. The cells were then allowed to differentiate into contractile myotubes for 6-7 days before use in experiments (Nedachi, Fujita, & Kanzaki, 2008; Park et al., 2008).

3.3 Electrical Stimulation of C2C12 Myotubes

Prior to stimulation, the medium was changed to 4 ml of fresh differentiation medium per well, and again 12 hours into the stimulation when applicable. The 6-well circular C-Dish plates were connected to a C-Pace EP cell culture stimulator (IonOptix, MA, USA). The lids of the plates were fitted with carbon electrodes which extended into each of the wells, contacting the medium, allowing current to flow through the medium in each well. Cells were stimulated at 10 V using 1 second trains of pulses (at 50 Hz), every other second, for 24 hours at 37 ^{o}C and 5% CO_2, causing contraction of the differentiated myotubes. After stimulation, the cells were extracted on ice, either immediately or after further incubation in fresh medium, as required for the experiment.

3.4 BSO Treatment

Endogenous GSH was depleted in C2C12 cells by the addition of BSO, an irreversible inhibitor of γ-GCS (key enzyme for GSH synthesis), to the differentiation medium at a concentration of 1 mM and 50 µM (Ardite et al., 2004). 6-well plates containing differentiated C2C12 myoblasts were washed with PBS and selected wells treated with differentiation medium containing the higher concentration (1 mM) BSO for 24 hours before stimulation. Control wells were incubated with standard differentiation medium. At the beginning of stimulation, the medium of all BSO treated wells was switched for the lower concentration (50 µM) BSO treatment medium. All control wells were given fresh medium immediately prior to stimulation.

The effectiveness of the BSO treatment at depleting GSH was determined using an Invitrogen glutathione fluorescent detection kit according to manufactures instructions and described below.

3.5 Glutathione Assay

Intracellular GSH was measured using a glutathione fluorescent detection kit (Invitrogen). In brief, both treated and control cells were extracted in ice-cold 5% SSA and lysed using multiple freeze thaw cycles and centrifugation (10 min at 14,000 rpm, Eppendorf 5424 R centrifuge, F-45-18-11 rotor), removing the supernatant after each cycle while leaving the pellet containing the lysed cells. Following incubation on ice, the pellet was centrifuged at top speed (21,130 × g). Pellet was collected and 25 µl of detection reagent was added in accordance to the manufacturer's instructions. The detection reagent covalently binds to the free thiol group of GSH, yielding a fluorescent product allowing the total amount of GSH to be assayed using a standard curve using an excitation wavelength of 390 nm and emission wavelength of 510 nm. An initial measurement allowed the detection of all free reduced GSH in the sample, while a second reading taken after the addition of the reaction mixture that reduced the remaining GSSG to GSH allowed the measurement of both GSSG and total GSH.

3.6 Cell Viability

C2C12 cell viability was measured at the end of the 24 hr stimulation period using the trypan blue exclusion technique (Strober, 2015). Cells were detached from 6-well plates using trypsin and resuspended in proliferation medium. 0.5 mL of Trypan Blue was added to 0.5mL of resuspended cells and the viable cells counted using a Countess II FL cell counter (Invitrogen, CA, USA). Cell counts were confirmed using a haemocytometer. The count was performed within 4 minutes of the cells exposure to trypan blue to avoid false positives. All cell viability results are expressed as percentage (%) of viable cells.

3.7 RT-PCR

Total RNA was extracted from C2C12 cells in 6-well plates using an Isolate II RNA Mini Kit according to the manufacturers protocol immediately after stimulation and one- and three-hours post-stimulation, as indicated in the results. Samples from three wells of each experimental group were pooled together and considered as one replicate. RNA quality (absorbance at 260/280 nm) and concentration were quantified using a Nanodrop Spectrophotometer (ThermoScientific, USA). cDNA was produced by reverse transcription using the high-capacity cDNA reverse transcription kit (ref:4368813) (ThermoScientific, USA). 1 µg total RNA was used as template according to the manufacturer's instructions.

Real-Time quantitative Polymerase Chain Reactions (RT-qPCR) were set up using a Universal SYBR Green One-Step kit from Applied Biosystems. Each well on the reaction plate contained: 0.1 µL forward primer, 0.1 µL reverse primer (10 µM final concentration), 3.8 µL nuclease-free water, 5 µL SYBR green, 1 µL cDNA, in a total volume per well of 10 µL. A full list of all primers used for this study can be found in Appendix 1. PCR reactions were performed using a StepOne RT-PCR instrument (Applied Biosystems) using the following parameters; samples heated to 95 °C for 5 minutes, 95 °C for five seconds followed by 60 °C for 30 seconds (repeated for 40 cycles), heated to 95 °C for 15 seconds, 60 °C for 1 minute, heated to 95 °C for 15 seconds.

RT-PCR analysis was performed using Thermo Fisher StepOne v2.3 software. Results were analysed using the $2^{-\Delta\Delta CT}$ method as described in Livak and Schmittgen (2001). This method of analysis allows for the measurement of a target gene's expression relative to a control and normalized by a housekeeping gene, in this case hypoxanthine-guanine phosphoribosyl transferase (HPRT).

3.8 ROS Assay (Hydrogen Peroxide)

ROS production was measured using a horseradish peroxidase (HRP)-linked Amplex UltraRed fluorescence assay (Invitrogen). The Amplex agent is specific for H_2O_2, this specificity is enhanced when in the presence of HRP as is used in this study. When Amplex UltraRed is oxidised by H_2O_2, catalysed by HRP, the reaction produces resorufin. This red fluorophore is stable and fluorescent when excited at 540 nm, emitting light at 612 nm. Accumulation of resorufin is proportional to the release of H_2O_2 into the medium. This allows the measurement of H_2O_2 produced within the cell which is released into the extracellular medium. C2C12 myotubes were incubated in PBS with added 25 mM glucose, 0.49 mM magnesium chloride and 0.9 mM calcium chloride, 0.1 U/mL HRP and 50 µM Amplex UltraRed. Half of the samples were also treated with 1 mM BSO for 24hr prior to, and then 50 µM directly before stimulation. A control was performed for each group using catalase at a final concentration of 300 U/ml. The catalase was added to one replicate in both BSO treated and untreated groups for each test plate. Fluorescence was measured identically to other samples. These results provide a background reading and are subtracted from the relevant sample group to produce the final readings. Samples were electrically stimulated as described previously. Aliquots of medium (100 µl) were taken from each of the wells containing differentiated C2C12 myotubes at 0, 15, 30 minutes, 1 and 3 hours into stimulation. Samples were transferred to a 96-well plate and the fluorescence measured (excitation 540 nm and emission 612 nm) using a FLUOstar OPTIMA fluorescence microplate reader (BMG Labtech, Ortenberg, Germany). H_2O_2 concentrations were calculated using a standard curve produced from known H_2O_2 concentrations (0.066 µM – 100 µM) and normalised to total cellular protein, measured by Bradford Assay (Bradford, 1976). Once the final sample had been taken for the Amplex readings, each well was then washed with 300 µl of ice-cold PBS and 300µL of a Triton Lysis Buffer (TLB) was added to extract the cells (20 mM HEPES, 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 0.5 mM DTT, 1 mM PMSF, 10% glycerol, 1% Triton X-100, pH 7.4). Bradford assays were performed in clear, 96-well flat-bottom plates. Each well contained 18µL of H_2O, 2 µL of sample, and 180 µL of Bradford reagent. The plate was incubated in the dark at room temperature for 5 minutes before the absorbance at 595 nm was measured from the underside of the plate. Protein concentrations were determined using a standard curve produced using known concentrations of bovine serum albumin (BSA) following the same protocol.

3.9 Statistical Analysis

Results are presented as mean ± standard error (SE). Two-way ANOVA was used to measure the overall effect of GSH depletion and ES on cell viability, GSH content of cells following BSO treatment, and on gene expression. The effect of GSH depletion on the accumulation of H_2O_2 over time during ES was analysed using repeated measures ANOVA. Bonferroni post-hoc test was used for specific inter-group comparison. Unpaired t-tests were performed to compare results of gene expression in control and GSH depleted cells at baseline (without ES). Results were analysed using Graphpad Prism 4 (v4.02, GraphPad Software Inc, La Jolla, CA, USA). The significance level was set at 5% ($p < 0.05$).

4. Results

In order to test if GSH had a measurable effect on the expression of genes involved in muscle adaptation to contraction, differentiated C2C12 myotubes were treated with BSO to deplete cellular GSH. Cell viability was determined in order to eliminate the possibility of non-viable cells skewing later results. Cells treated with BSO were compared to control cells to confirm that the treatment had successfully reduced GSH levels. BSO treated cells along with control groups were electrically stimulated to induce contraction. H_2O_2 content and mRNA expression were measured in order to determine the effects of depleting endogenous GSH in C2C12 myotubes.

4.1 Experimental Groups

In order to evaluate the importance of GSH in the response to contractile activity in skeletal muscle, cells were treated with BSO before and during ES. This treatment was used to deplete GSH content in the cells before, during, and after the stimulation period. The experimental design is summarised in Figure 6.

The experimental groups were defined as the following, all groups use differentiated C2C12 myoblast cells:

- Control Baseline: Cells in standard medium. No electrical stimulation.
- Control ES (electrical stimulation): Cells in standard medium submitted to electrical stimulation to elicit an exercise response.
- GSH Depleted Baseline: Cells treated with BSO to allow for analysis of cellular response and changes caused by a lack of GSH. No electrical stimulation.
- GSH Depleted ES: Cells treated with BSO and submitted to the ES protocol for analysis of exercise response in the absence of GSH.

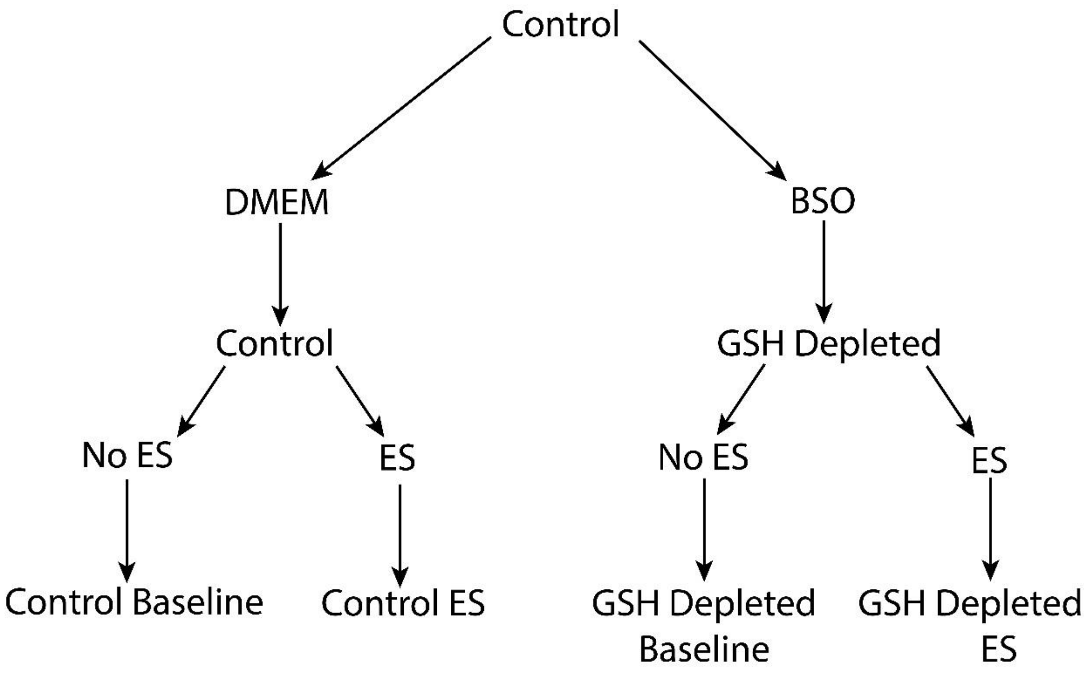

Figure 6. Basic outline of the experimental groups used in this study. These groups are used primarily when referring to the RT-PCR results. Addition of BSO to the culture medium significantly depletes cellular levels of GSH, while DMEM was used as a control medium. Electrical stimulation (ES) was performed as described above. Baseline groups were those which did not undergo any form of ES and were used as an "at rest" group.

4.2 Cell Viability

In order to test if C2C12 myotubes remained viable following BSO treatment and electrical stimulation cells from all experimental groups were stained with trypan blue and counted using a Countess II. This method uses the exclusion technique as described by Strober (2015). Non-viable cells take up the trypan blue dye and show up as a distinct dark blue colour while viable cells exclude the dye and appear a lighter blue. Non-viable cells would cause later results to be unreliable due to varying H_2O_2 release, GSH content and altered mRNA measurements.

Cell viability tests were conducted on all experimental groups, as outlined above. The results, shown in Figure 7, indicate that neither GSH depletion through treatment with BSO ($p = 0.0978$; two-way ANOVA) nor electrical stimulation as per protocol ($p = 0.7654$; two-way ANOVA) had any significant effect on the viability of the differentiated C2C12 myotubes used in this experiment. Total cell survival rates had a mean of 96.9% ± 0.3% across all experimental groups. Control baseline cells, which were not depleted of GSH nor subject to electrical stimulation, had a mean survival rate of 95.6% ± 0.9% and control ES was similar at 96.8% ± 0.7%. Mean survival rate of the GSH depleted baseline group was 98.5% ± 0.3% and GSH depleted ES was 96.8% ± 0.5%. These results show that neither the electrical stimulation protocol nor treatment with BSO, alone or combined, cause any significant reduction in cell viability when compared to controls.

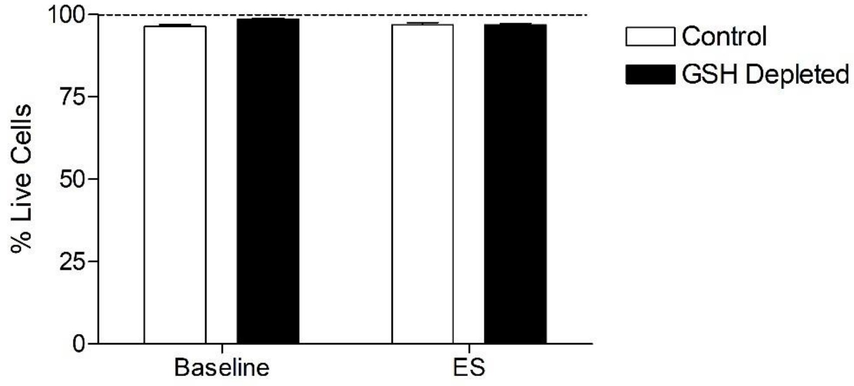

Figure 7. Cell viability (% of live cells) of GSH depleted (black) and control (white) cells at baseline and following ES. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. p = 6-12 for all groups. Number of cells/well was similar among groups: $5.5x10^5$/mL ± $0.6x10^5$/mL.

4.3 GSH Depletion in C2C12 myotubes

GSH had to be significantly depleted in groups of cells in order to test the effects of GSH on gene expression. This allows a comparison to be drawn between GSH depleted cells and control cells where GSH levels are at normal physiological levels. BSO was used to achieve this and, to test the effectiveness of this chemical inhibitor of GSH synthesis, total and free GSH levels of cells were measured. Measurements were taken from groups of cells which were treated with BSO (GSH depleted) and those which had not (control). Cells from both groups were then subjected to ES and compared to baseline (unstimulated) cells of the same group.

As shown in Figure 8, treatment with BSO caused a significant decrease in cellular GSH when comparing total GSH concentrations of treated (GSH depleted) and untreated (control) cells across both baseline and ES groups (collected immediately after ES). Mean total GSH concentrations were 36.4 µM ± 7.8 µM and 36.8 µM ± 9.3 µM for baseline and ES control cells respectively. GSH depleted cells had mean concentrations of 5.6 µM ± 0.9µM for baseline and 6.8 µM ± 0.9 µM for ES cells, significantly lower concentrations ($p < 0.001$; Bonferroni post-test) when compared to control cells showing that BSO was effective at depleting GSH. ES was shown to not have a significant effect on total GSH content immediately after the protocol ($p = 0.8740$; two-way ANOVA).

These results show that the BSO treatment used in this study was effective in significantly depleting the total cellular GSH in C2C12 myotubes.

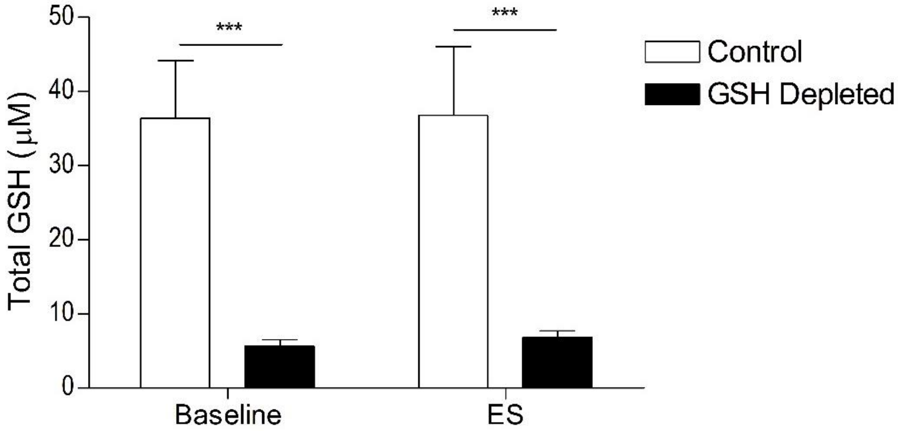

Figure 8. Depletion of GSH in C2C12 myotubes. Total GSH concentration (μM) per well of control cells (white) and those treated with BSO (black) at baseline and immediately following ES. *** $p < 0.001$, Bonferroni post-test. Number of cells: 5.5×10^5/mL ± 0.6×10^5/mL. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. n = 7-12 for all groups.

4.4 H_2O_2 Release from C2C12 myotubes Measured by Amplex Red Fluorescence

As endogenous GSH plays a key role in the elimination of H_2O_2 within C2C12 myotubes, further tests were conducted in order to determine if GSH depletion caused additional H_2O_2 accumulation when compared to controls. Measurements were taken of C2C12 myotubes treated with BSO (GSH depleted) and control cells. Some cells from each group underwent contraction via ES while some did not (baseline).

GSH depletion significantly increased H_2O_2 accumulation, both at baseline and during ES ($p = 0.0076$; repeated measures ANOVA). ES enhanced H_2O_2 production over time when compared to non-stimulated cells ($p < 0.001$; repeated measures ANOVA), with GSH depletion causing a further increase in H_2O_2 production, in addition to ES, when compared to untreated cells ($p < 0.0057$; repeated measures ANOVA) (Figure 9).

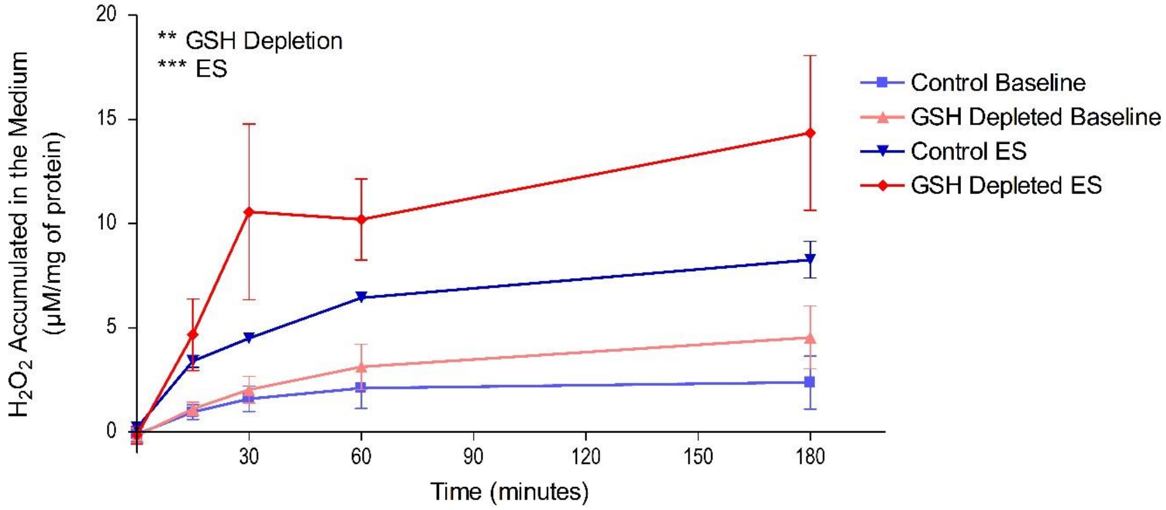

Figure 9. Change in accumulated H_2O_2 concentration over time in medium from C2C12 myotubes. H_2O_2 measurement of GSH depleted and control cells during ES and at baseline (no ES). ** $p < 0.01$ for an overall effect of GSH depletion on H_2O_2 concentration over time (repeated measures ANOVA). *** $p < 0.001$ for an overall effect of ES on H_2O_2 concentration over time (repeated measures ANOVA). Data presented as mean ± SE. Data analysed by repeated measures ANOVA and Bonferroni post-test. n = 4 for all groups.

4.5 Gene Expression Measured by RT-PCR

To determine the effect of GSH depletion on contraction-induced changes in gene expression, groups of GSH depleted and control cells were subjected to electrical stimulation while others were left in a resting state (baseline). The mRNA levels of genes were measured using RT-PCR in both GSH depleted and control cells, at three time points (0 hr, 1 hr and 3 hrs) following contraction along with a baseline group which was not subjected to ES. The results are divided into sections; the first grouping genes which showed no response following ES, the second displaying genes, which had expression changes at baseline, and finally genes for which mRNA levels changed following ES or GSH depletion.

4.5.1 Genes Not Affected by GSH depletion or ES (CS, HK, GPx1, Tfam, NRF1, NRF2, SOD2)

The genes for CS, HK, GPx1, Tfam, NRF1, NRF2 and SOD2 showed no significant change in mRNA expression over time following stimulation, nor did the response vary significantly between GSH depleted and control cells (Figures 10, 11). Although Tfam had a greater increase in mRNA when compared to other non-responding genes, this increase was still not statistically significant (p = 0.36 for and effect of ES over time, p = 0.61 for and effect of GSH depletion; two-way ANOVA).

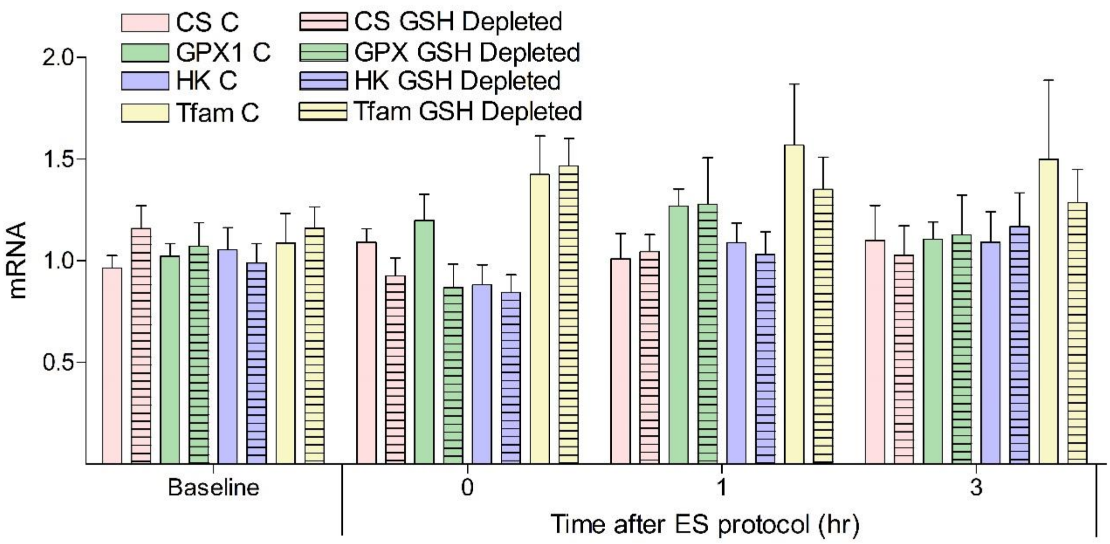

Figure 10. mRNA levels of genes not affected by GSH depletion or ES measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. Solid bars = Control, Striped bars = GSH depleted. n = 11 for all genes. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

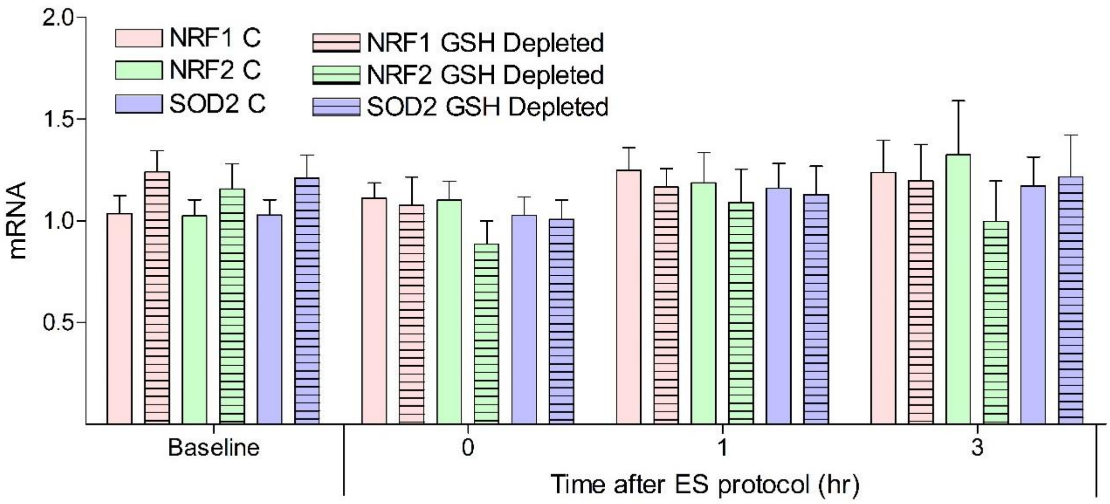

Figure 11. mRNA levels of genes not affected by GSH depletion or ES measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. Solid bars = Control, Striped bars = GSH depleted. n = 11 for NRF1 and SOD2. n = 9 for NRF2. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

4.5.2 Baseline Effects of GSH Depletion (PPARα, PFK and SOD1)

The mRNA levels of PPARα, PFK and SOD1 were measured using RT-PCR as described in the methods. These genes showed no significant changes in expression when comparing GSH treated cells and controls overall or at any specific timepoint following the electrical stimulation protocol used in this study (Figure 12A). However, at baseline all three showed a tendency (although not statistically significant) for GSH depleted cells to have higher expression (SOD1 $p = 0.086$, PPARα $p = 0.0654$, PFK $p = 0.1149$; unpaired t-test) (Figure 12B). The GSH depleted values are higher by 36% for SOD1, 27% for PPARα and 34% for PFK. These results may suggest that GSH affects the expression of SOD1, PFK, and PPARα at baseline already before cells are subjected to electrical stimulation.

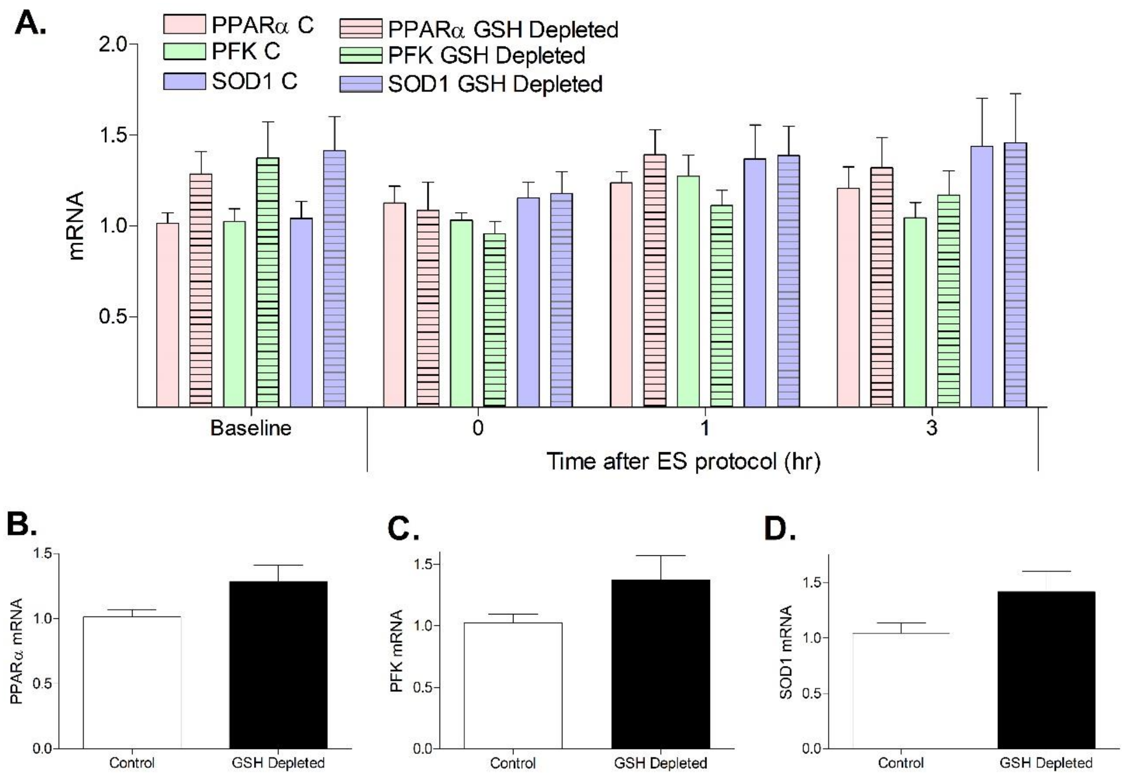

Figure 12. mRNA levels of PPARα, PFK and SOD1 measured by RT-PCR. **A.)** Gene expression at baseline and following ES of PPARα, PFK, and SOD. Data analysed by two-way ANOVA and Bonferroni post-test. **B.)** Baseline expression of PPARα; $p = 0.0654$ (unpaired t-test). **C.)** Baseline expression of PFK; $p = 0.1149$ (unpaired t-test). **D.)** Baseline expression of SOD1; $p = 0.086$ (unpaired t-test). Data presented as mean ± SE. Data analysed using an unpaired t-test. $n = 11$ for PFK and SOD1. $n = 9$ for PPARα. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

4.5.3 Genes Affected by Interventions (VEGF, CPT-1B, PPARγ, Catalase, GLUT4, PGC-1α)

The mRNA levels of genes were analysed using both two-way ANOVA and Bonferroni post-tests. Significant overall effects ($p < 0.05$) were found for the genes in this section using the two-way ANOVA. These effects relate to overall changes in mRNA levels either over time following ES (*time) and/or the effect of GSH depletion following treatment with BSO (*GSH depletion) when compared to baseline (unstimulated) untreated cells. No significant differences were found when comparing between specific groups using the Bonferroni post-test.

4.5.3.1 VEGF

VEGF expression increased over time following ES for both control and GSH depleted cells. Two-way ANOVA analysis showed a significant response for an effect of time following ES ($p = 0.0394$). There was no significant difference in VEGF mRNA levels in GSH depleted myotubes compared to controls at any time point ($p = 0.4459$; two-way ANOVA). VEGF mRNA levels peaked at the one-hour time point for both GSH depleted and control cells with increases of 11.3% and 26.1% respectively, before both decreased approximately back to baseline values at the three-hour time point (Figure 13). These RT-PCR results show that VEGF mRNA increases following electrical stimulation, whether or not endogenous GSH has been depleted.

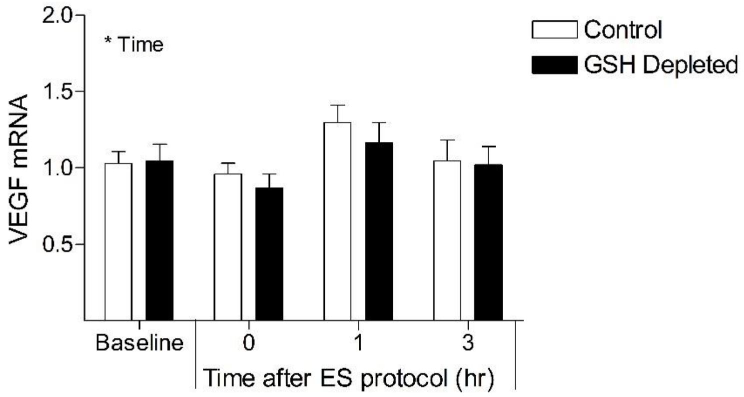

Figure 13. VEGF mRNA levels measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni test. *$p < 0.05$ for an overall effect of ES over time. n = 11. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

4.5.3.2 CPT-1B

CPT-1B expression was measured in GSH depleted and control cells at baseline and at multiple timepoints following a 24-hour electrical stimulation period as described in the methods. There was a strong, although not statistically significant, tendency for CPT-1B expression to increase over time following ES ($p = 0.0580$; two-way ANOVA) with no difference between control and GSH depleted groups ($p = 0.1732$; two-way ANOVA). There was an increase in CPT-1B mRNA expression from the baseline reading to the one-hour timepoint for GSH depleted cells, before a decrease to baseline levels at the three-hour time point. GSH depleted cells peaked at the one-hour time point with an increase of 56.3% while control peaked after three hours with an increase of 32.8%, although this was similar to the zero and one-hour values of 32.3% and 32.7% respectively (Figure 14). Following electrical stimulation CPT-1B expression increased over time and was unaffected by the depletion of endogenous GSH.

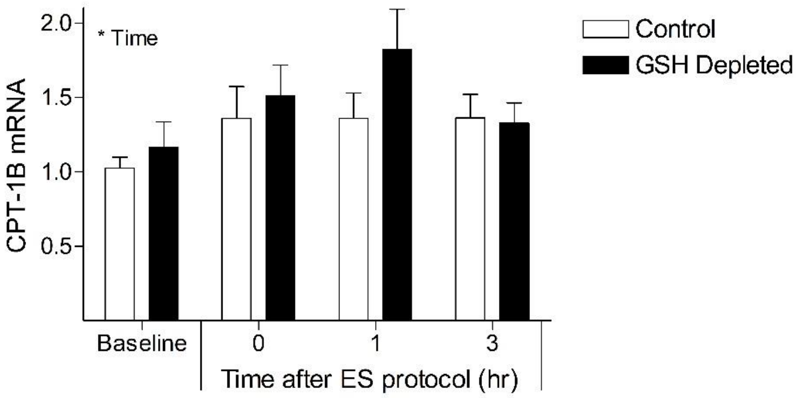

Figure 14. CPT-1B mRNA levels measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test; *$p = 0.0580$ for an overall effect of ES over time. n = 11. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

4.5.3.3 PPARγ

GSH depletion caused an increase in PPARγ expression when all time points are considered ($p = 0.0427$; two-way ANOVA). This effect was greatest at baseline and the three-hour post-stimulation timepoint where PPARγ expression was 22.2% and 31.9% higher in GSH depleted samples in comparison to controls, respectively. ES had no significant effect on PPARγ mRNA levels as no large changes in expression were seen when comparing baseline results to any timepoint following stimulation. ($p = 0.7622$; two-way ANOVA) (Figure 15). These results show that depleting endogenous GSH of

C2C12 myotubes causes a baseline increase in PPARγ mRNA levels which persists following contraction.

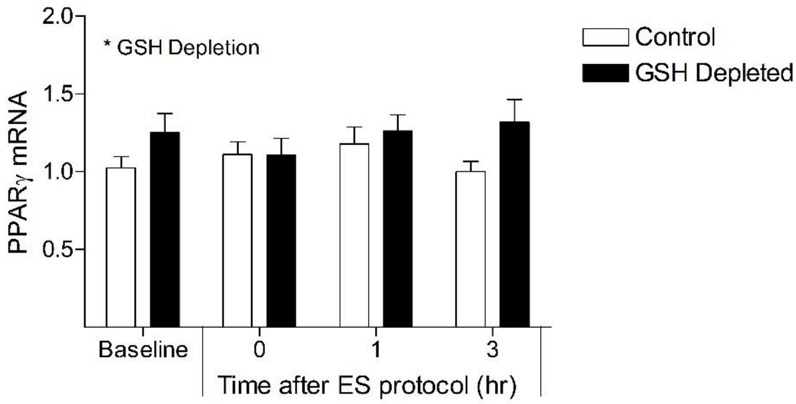

Figure 15. PPARγ mRNA levels measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. *$p < 0.05$ for an overall effect of GSH depletion. n = 11. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

4.5.3.4 Catalase

Catalase expression was measured in GSH depleted and control myotubes at baseline and at multiple timepoints following a 24-hour electrical stimulation period as previously described in the methods. Catalase expression was upregulated by GSH depletion and, at the same time, was increased by ES. Untreated control cells also showed an increase in catalase mRNA from baseline to the one-hour timepoint (45.4%), before flattening off to the three-hour point remaining 23.3% higher at the three-hour point than at baseline. GSH depleted cells also showed a steady increase in catalase expression from baseline to the three-hour point peaking at 43.3% above baseline. However, mRNA levels of GSH depleted cells were higher than that of control cells at every timepoint. Statistical analysis showed a significant effect of both ES (time) and treatment (GSH depletion) over the entirety of the time course with values of $p = 0.0027$ and $p = 0.0281$ respectively (two-way ANOVA). (Figure 16). These results show that the depletion of endogenous GSH of C2C12 myotubes causes an increase in catalase mRNA levels both at baseline and following contraction, while the responsiveness of catalase to ES remains similar regardless of GSH levels.

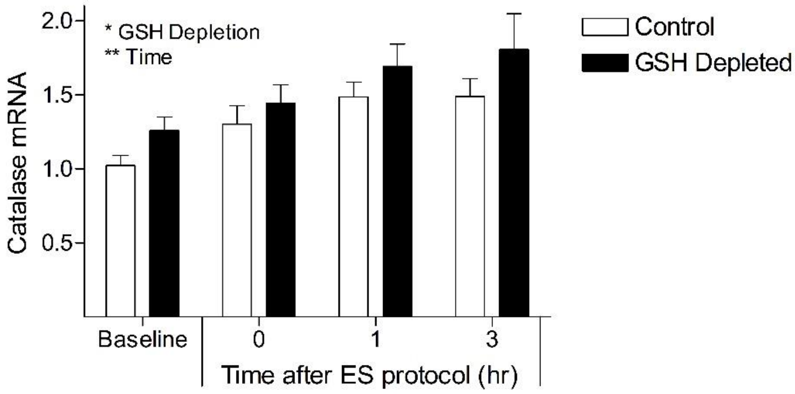

Figure 16. mRNA levels of catalase measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. *$p < 0.05$ for an overall effect of GSH depletion. **$p < 0.01$ for an overall effect of ES over time. n = 11. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

4.5.3.5 GLUT4

There was a significant effect of ES on the expression of GLUT4 in both GSH depleted and control cells ($p = 0.0016$; two-way ANOVA). GLUT4 expression increased over time following electrical stimulation for both test groups, regardless of GSH levels cells ($p = 0.3369$; two-way ANOVA) (Figure 17). At the three-hour post-ES time point both groups saw their peak in GLUT4 expression, increasing from baseline 108% for GSH depleted and 113.7% for control cells. These results show that GSH depletion has no effect on GLUT4 mRNA, while electrical stimulation caused an increase in GLUT4 expression over time following contraction.

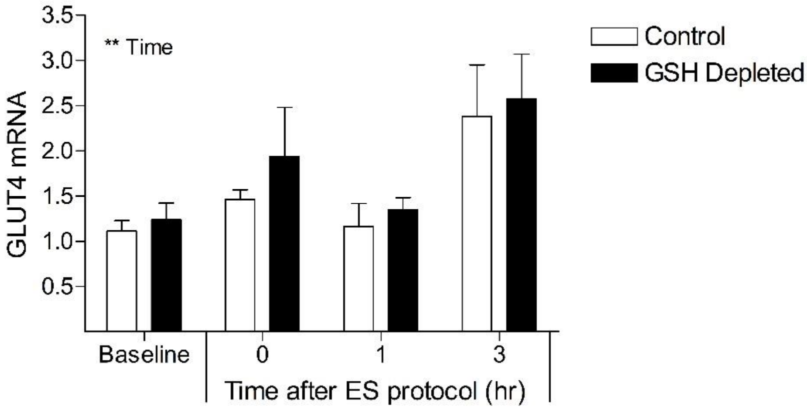

Figure 17. GLUT4 mRNA levels measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. **$p < 0.01$ for an overall effect of ES over time. n = 11. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

4.5.3.6 PGC-1α

Two-way ANOVA analysis showed a significant effect of ES on the expression of PGC-1α across both GSH depleted and control groups when all timepoints are considered ($p = 0.0249$; two-way ANOVA). PGC-1α expression increased over time following ES for both GSH depleted and control groups alike cells ($p = 0.7323$; two-way ANOVA). GSH depleted cells showed an increase in PGC-1α gene expression from baseline to three hours post-ES, peaking 43.6% higher after 1 hour, before decreasing to the final timepoint (+27.4% from baseline). Control groups where GSH production was not impaired showed an increase of 75% (1.032-1.798) from baseline to its peak at the zero-hour point, before decreasing at a slower rate with a final reading still of 1.494, 44.8% above baseline. (Figure 18). These results show electrical stimulation increases PGC-1α mRNA levels in C2C12 myotubes. GSH depletion had no significant effect on PGC-1α mRNA as expression did similarly increase following electrical stimulation, regardless of treatment.

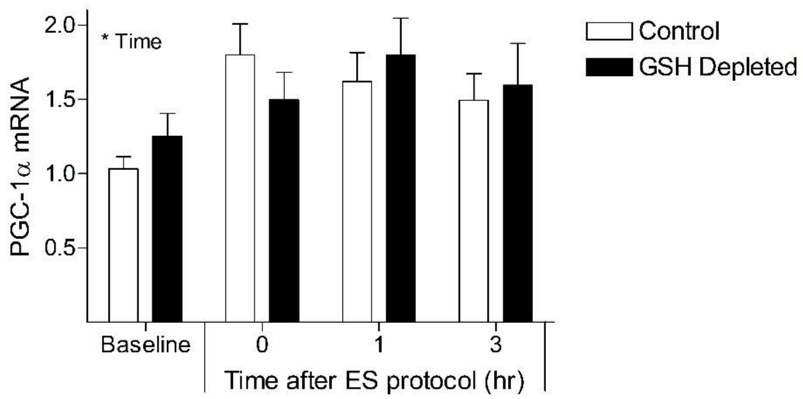

Figure 18. PGC-1α mRNA levels measured by RT-PCR. Data presented as mean ± SE. Data analysed by two-way ANOVA and Bonferroni post-test. *$p < 0.05$ for an overall effect of ES over time. n = 11. Data was normalised to the control baseline of each gene. HPRT was the housekeeping gene for all measurements.

5. Discussion

5.1 Overall Effects

This study aimed to evaluate the involvement of endogenous GSH in the molecular adaptation of skeletal muscle myotubes to contractions induced by ES *in vitro*. The results gathered suggest that there is no marked effect of GSH depletion on gene expression following contraction (Figure 19). Previous studies have shown the effects of ROS on signalling pathways involved in muscle adaptation following exercise, but the current study focused on the potential role of GSH in this signalling. The current results suggest that although GSH may not hold the key to ROS mediated muscle adaptation, it still plays an important role in controlling the redox state of cells and may influence gene expression through a mechanism that has not yet been elucidated.

Despite observing a significant difference between the levels of GSH and H_2O_2 produced in control and cells treated with BSO (blocking GSH synthesis), no measurable difference was observed in the expression of markers of muscle adaptation following ES. All cells were treated to similar proliferation and differentiation times allowing for myotubes to form before treatment and stimulation took place. Other factors may have a more significant effect on adaptation signalling in comparison to the involvement of GSH. Therefore, although the depletion of GSH may have had an effect on the signalling involved in muscle adaptation, it may not have been of sufficient magnitude to be detectable without the suppression of other, more prominent, signalling pathways activated during exercise, such as those involving AMPK, MAPK, CaMK or NF-κB. Additionally, due to the minimal changes in expression observed following muscle contraction the ability to ascertain the role of GSH in these changes is limited.

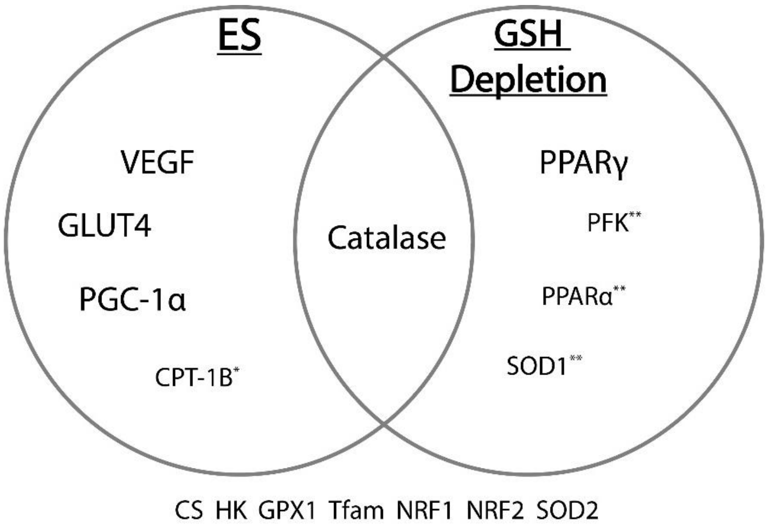

Figure 19. The effects of GSH depletion and ES on genes for which mRNA expression increased following electrical stimulation (ES), GSH depletion or both, as measured by RT-PCR. Genes represented outside of both circles were unaffected. *p = 0.058. **Effect at baseline only; SOD1 p = 0.086, PPARα p = 0.0654, PFK p = 0.1149.

5.2 The Effect of BSO on ROS Generation in Muscle

The depletion of GSH through inhibition of γ-GCS by BSO on differentiated C2C12 myotubes resulted in a significant reduction of total GSH levels. This is in line with previous studies and was consistently reproduced over time (Ardite et al., 2004; Griffith & Meister, 1979; Savini et al., 2005). GSH acts as an antioxidant molecule and is one of the main scavengers of H_2O_2 in skeletal muscle (Blokhina et al., 2003; Meister & Anderson, 1983; Pizzorno, 2014). Therefore, the lack of GSH is likely to reduce myotube antioxidant potential, inhibiting the ability of GPx to catalyse the oxidation of GSH by H_2O_2.

Control cells that were not subjected to contraction were able to stabilise the H_2O_2 signal after one hour, while GSH depleted cells continued to release H_2O_2 causing an increase in the Amplex signal over time. This may illustrate the importance of GSH on the redox balance of muscle cells even at rest, although it was not possible to evaluate the effect GSH depletion had on other antioxidant molecules. A wide range of other antioxidants contribute to the maintenance of intracellular redox status, including enzymes such as catalase and SOD1/2 as well as molecules such as peroxiredoxin and glutaredoxin. Although GSH is the most abundant of these, together the other antioxidants may be capable of controlling H_2O_2 levels under baseline conditions, irrespective of whether GSH is depleted.

This study demonstrates the increase in ROS production during contraction, like many others described (Davies et al., 1982; Powers et al., 2011; Reid, 2001b). The accumulation of H_2O_2 from GSH depleted cells was even more prominent during ES than at baseline. Cells where GSH levels were lowered produced a significant increase in H_2O_2 during ES, greater than that seen by ES alone. This indicates that these myotubes were less able to balance H_2O_2 production and elimination in comparison to control cells. This emphasises the importance of GSH (or the GSH-GPx-GR system) and its role in maintaining the redox balance of muscle.

5.3 Mitochondrial Biogenesis and Fatty Acid Metabolism

Mitochondrial biogenesis and fatty acid metabolism are controlled by a similar range of signalling pathways and transcription factors. Controlling the expression of transcription factors such as PPARα and PGC-1α, the AMPK pathway plays a central role in regulating mitochondrial biogenesis and fatty acid metabolism in muscle cells. AMPK in known to be sensitive to AMP/ADP ratios within the cell but may also be stimulated by H_2O_2 (Hinchy et al., 2018). GSH depletion significantly increased concentrations of H_2O_2, which may have led to an increase in the expression of PPARα mRNA in cells prior to contraction when compared to controls. While this increase was clear at baseline, following contraction there was no significant difference in PPARα expression. This is in contrast to PGC-1α mRNA expression which increased over time following contraction yet GSH depletion did not alter this response further. These inconsistencies between genes at baseline and following ES are likely due to the variety and crosstalk of signalling pathways activated by ROS and ES. Interestingly, PGC-1α peaked at zero hours in control cells while GSH depleted cells peaked one hour following stimulation. Although there is no statistical significance, along with the lack of more prominent response of PGC-1α to stimulation, this difference shows a possible influence of GSH depletion on the level of PGC-1α expression following contraction which cannot be ruled out.

As with many genes measured in this study, other signals, such as those triggered by exercise, are likely to affect the expression of PGC-1α. Shown in Figures 3 and 4, a wide range of factors directly and indirectly influence PGC-1α expression, stability and activity. Since these pathways were not monitored in this study, it is possible that they may contribute to PGC-1α regulation, alongside H_2O_2 and GSH. PGC-1α expression is controlled by multiple upstream activators which act independently of ROS, activated by signalling trough Ca^{2+}, cAMP, AMP and more. The measurement of these pathways including p38 MAPK, CaMKII, AMPK, PKA, and calcineurin may provide further insight into the different signalling pathways activated in GSH depleted C2C12 cells compared to controls. This would also provide a clearer insight into PGC-1α's role in the expression of other genes involved in all aspects of muscle adaptation following contraction.

Much like PGC-1α and PPARα, the expression of CPT-1B mRNA after ES was not affected by GSH depletion and, similar to PGC-1α, there was a significant increase in CPT-1B expression following contraction, regardless of GSH levels. CPT-1B dictates the rate of β-oxidation and its expression is known to be linked to AMPK, as well as p38 MAPK. Further research may provide an explanation for the small difference between control and GSH depleted groups if this is mirrored in the activity of either AMPK or p38 MAPK signalling (Ronnett, Kleman, Kim, Landree, & Tu, 2006). Results from the current study do not provide any evidence to suggest a significant effect of GSH depletion on CPT-1B expression.

Although the majority of genes involved in the adaptation of fatty acid metabolism following contraction were unaffected by GSH depletion, PPARγ and PPARα showed significant changes in expression. PPARγ mRNA expression did not respond to contraction but was increased in GSH depleted groups when compared to controls. Similarly, PPARα mRNA expression increased at baseline in response to GSH depletion, while no change was seen when comparing control and GSH depleted groups following contraction. These results suggest that some aspects of fatty acid metabolism may be affected by either GSH depletion or the resulting increase in H_2O_2 during rest. Although PPARγ and PPARα are both known targets of PGC-1α activation, the increased expression of PGC-1α over time following ES did not correlate with changes in PPARγ or PPARα mRNA levels and may be a result of other signalling pathways activated by ROS and contraction.

There is a clear link between fatty acid metabolism and mitochondrial biogenesis in skeletal muscle. One of the aims of this study was to investigate the effect of GSH depletion on genes involved in the adaptive changes to these processes both at rest and in the exercise response. The results described above suggest that GSH depletion has no significant effect on these adaptations following contraction, although further investigation is required to elucidate the mechanisms responsible for changes in PPARγ and PPARα expression at rest after GSH depletion.

5.4 Glucose Metabolism

The results in this study also suggest that increased GLUT4 expression in response to ES is not impaired by GSH depletion, as both control and GSH depleted cells showed a significant increase in expression of GLUT4 over time following ES. Both groups followed a similar trend, peaking first immediately following ES and then again after three hours. This is in line with similar studies which showed little increase in GLUT4 immediately after ES followed by a significant increase in the subsequent hours (Kuo, Browning, & Ivy, 1999).

Although GLUT4 expression has previously been shown to be stimulated by ROS (Katz, 2016; Pinheiro, Silveira, Nachbar, Vitzel, & Curi, 2010), results from the current study show no significant increase at baseline where ROS is increased in GSH depleted cells. A wide range of signals elicited by exercise such as activation of AMPK, MAPK, and CaMK are known to affect GLUT4 expression (Holmes et al., 2005; Kuo et al., 1999; Ojuka, Jones, Nolte, et al., 2002). These pathways are mainly active during stimulation but are also redox sensitive, and therefore may be activated by the elevated levels of ROS in GSH depleted cells although no such effect was observed in this study. Analysing the activation of these pathways may provide some insight into the many factors controlling GLUT4 expression both at baseline and following ES.

GSH depletion increased PFK expression prior to contraction when compared to control cells. This suggests that the lack of GSH or increase in H_2O_2, at least for a short duration (i.e., 24 hours) may have beneficial effect on glucose metabolism in the muscle cells. PFK was the only gene tested that is directly involved in glucose metabolism and displayed any change following GSH depletion. Although PGC-1α is involved in the regulation of glucose metabolism in muscle, it only displayed an increase as a result of muscle contraction, as did GLUT4. Therefore, the interplay between ROS and exercise appear to be important to improve glucose metabolism, while reduced levels of GSH for a short period have no effect on the metabolic indicators used in this study.

5.5 Antioxidant Capacity

Muscle cells which have significantly lowered antioxidants would be expected to produce higher levels of ROS during exercise. This has been shown in studies where GSH levels were depleted in skeletal muscle and ROS levels, such as H_2O_2, increased when compared to controls following exercise (Dam, Mitchell, Rush, & Quadrilatero, 2012). Similarly, in GSH depleted cells, the activity of other antioxidants in the cell could increase and compensate for the reduced GSH. The activity of antioxidant enzymes such as GPx1 and SOD2 are known to be tied to the expression of PGC-1α, which was not affected by GSH depletion, and other signalling pathways, such as NF-κB and MAPK. NF-κB signalling has been shown to be both increased and decreased by ROS, although the most recent evidence supports NF-κB as being activated by exercise-induced ROS (Ji, 2008; Ji et al., 2007; Kabe, Ando, Hirao, Yoshida, & Handa, 2005; Pantano, Reynaert, van der Vliet, & Janssen-Heininger, 2006). The PGC-1α promotor contains a binding site for NF-κB, suggesting that ROS produced during contraction may lead to PGC-1α expression via NF-κB signalling.

Although ROS, PGC-1α and NF-κB activation being implicated in the expression of GPx1 and SOD2, the current study showed no response of SOD2 and GPx1, despite changes in H_2O_2 levels and PGC-1α expression. This highlights the complex crosstalk between signalling pathways activated by ROS and exercise that defines gene expression. Our findings are partially in contrast to findings from a previous study by Strobel et al. (2014). In line with the current study, Strobel et al. (2014) showed no change in SOD2 expression following ES in control and GSH depleted groups. However, their results also showed an increase in GPx1 following exercise in control groups while expression decreased in GSH depleted groups, contrasting the results from current study. The Strobel et al. (2014) study used live animal models along with an alternative method of depleting muscle glutathione (diethyl maleate). These differences in findings may be due to variation in protocol between the studies.

Interestingly, multiple genes showed changes in expression at baseline when no contraction was undergone. One of these was the tendency for SOD1 mRNA to be increased in GSH depleted cells ($p = 0.086$). This is likely due to the increased levels of ROS and oxidative stress at baseline resulting from the lower levels of the key antioxidant GSH. This is supported by a previous study which showed that the promoter activity of the SOD1 gene is increased by H_2O_2 in skeletal muscle (Yoo, Chang, & Rho, 1999). The lack of a difference in SOD1 mRNA levels between control and GSH depleted cells following exercise may be due to the upregulation of other antioxidant mechanisms within the cell, such as catalase.

The expression of catalase was increased following both GSH depletion and ES, with incremental effects. Catalase mRNA was significantly higher in GSH depleted cells than control groups across all time-points, including baseline. Despite that, GSH depletion did not improve the responsiveness of catalase to ES, as the magnitude of increase in gene expression was similar between GSH depleted cells and control. Catalase expression in skeletal muscle is known to be controlled, in part, by the activation of p38 MAPK. The significant increase in catalase mRNA seen following both ES and GSH depletion may therefore be a result of p38 MAPK activation. Exercise is known to induce p38 MAPK signalling in skeletal muscle (Goodyear, Chang, Sherwood, Dufresne, & Moller, 1996). A previous study suggested that p38 MAPK is a key step in controlling the upregulation of catalase following increased levels of H_2O_2 (Sen, Chakraborty, & Raha, 2005). This study exposed lung fibroblasts to low levels of H_2O_2, resulting in increased p38-MAPK and catalase expression, while groups with silenced p38MAPK signalling, attenuated the increase in catalase expression. The decreased catalase expression observed in the study provides further evidence of a link between p38-MAPK activation and catalase expression. P38-MAPK may have been activated in the cells in the current study resulting in increased catalase mRNA.

However, those are not the only mechanisms involved in catalase expression. Independent of contraction and ROS signalling, Luo and Rando (2003) suggests that the transcriptional regulator NF-Y is a strong inducer of catalase expression, binding to two CCAAT boxes in the promoter of the gene, an effect that may be muscle cell specific and with an unclear mechanism of stimulation. It is therefore possible that NF-Y may be responsible for the increased activation of catalase seen in the current study. Other studies have shown that mutations to the CAAT boxes present in the human catalase gene significantly decrease its activation, although this was carried out on HP100 myeloid leukemia cells.

PGC-1α is also known to regulate catalase expression (St-Pierre et al., 2006). In the current study, PGC-1α and catalase expression showed very similar trends in GSH depleted cells, steadily increasing from baseline to the 1-hour timepoint (Figures 16 and 18). The similarity of these results and those of St-Pierre et al. (2006) indicate that PGC-1α may be, in part, responsible for the increased expression of catalase when GSH is absent.

GSH depletion may have reduced antioxidant capacity of the C2C12 myotubes, as displayed by greater H_2O_2 at baseline and during contraction. On the other hand, the lack of GSH caused an increase in the expression of catalase and SOD1, with no detrimental effects over the response of the genes to exercise. This suggests that GSH may be involved in the regulation of some antioxidant genes, although it is not necessary to elicit normal responses to elevated ROS or contraction. Furthermore, the increase in antioxidant gene expression at baseline was not great enough to counterbalance the lack of GSH, as ROS levels were consistently higher in the absence of GSH. Thus, long-term depletion of GSH, such as is seen in type 2 diabetes (Calabrese et al., 2012), may promote chronic oxidative stress and damage to cellular structures, becoming a relevant pathological factor of diseases.

5.6 Angiogenesis

The current study found that expression of VEGF mRNA in C2C12 cells increased over time following ES with no additional effect of GSH depletion. VEGF is known to be transcriptionally controlled by a range of pathways which can be activated by exercise, including p38 MAPK, ERK, and the PI3k-Akt pathways (Karar & Maity, 2011; Pagès & Pouysségur, 2005; Pogozelski et al., 2009). VEGF expression is also regulated by PGC-

1α following exercise (Leick et al., 2009). Therefore, the increase in VEGF expression observed following stimulation may be due, at least in part, to the changes seen in PGC-1α expression.

Previous studies have suggested that VEGF may be redox sensitive with its expression being stimulated by ROS (Kosmidou, Xagorari, Roussos, & Papapetropoulos, 2001). Results from the current study suggest that VEGF expression in skeletal muscle is not affected directly by a lack of GSH nor by a perturbed redox state due to increased H_2O_2 concentrations, but measurement of the activity of the pathways mentioned above could shed more light on the regulation of VEGF seen in this study.

6. Limitations and Future Directions

Although some significant changes in gene expression were measured in this study following contraction and GSH depletion, the pathways involved or mechanisms which brought about these changes were not determined. This is due to the use of RT-PCR as the primary measure of effect. Although this measures changes in mRNA levels, the effects of GSH depletion and contraction are likely to be much broader. Changes in protein expression, measured through Western Blotting, and activity of key enzymes would broaden the scope of this study and provide further insight into the mechanisms and pathways involved in the changes in mRNA reported herein. MAPK, CaMK, NF-κB, activator protein-1 (AP-1) and AMPK pathways are likely to play a significant role and their measurement would likely shed further light on the involvement of GSH in muscle adaptation following contraction. Furthermore, the monitoring of functional changes in enzyme activity, glucose uptake, glucose oxidation, and fatty acid oxidation would provide a clearer picture of the effects of GSH depletion on wider cell function. Additionally, experiments involving the measurement of the extent of S-glutathionylation of these proteins would help to identify if any changes in activity were due to direct modification by GSH.

The size of effects on gene expression brought about by the stimulation protocol used in this study the ability to compare the effects of GSH depletion on cells which had been subjected to contraction with those which had not.

The inclusion of additional control groups through treatment of GSH depleted cells with GSH reduced ethyl ester (GSHEE) would provide more robust evidence of the involvement of GSH in adaptation following exercise. GSHEE is permeable to the cell membrane and can be used to deliver GSH and restore physiological levels of intracellular GSH in myotubes treated with BSO. Any changes that occur in GSH depleted cells which are the direct result of a lack of GSH should return to levels measured in control cells. This would provide further evidence that changes in gene expression are in fact due to a lack of GSH and less likely a result of other indirect factors.

In the current study H_2O_2 was used as a marker of overall redox status of myotubes with only the level of H_2O_2 released from cells being measured. This applies another limitation on the study as without the measurement of the specific sources of ROS or the compartmentalised redox status, it is not possible to obtain a clear picture of the specific redox pathways being activated or supressed.

Contraction is known to activate many signalling pathways in C2C12 cells and therefore causes changes in gene expression not directly linked to redox stress. To isolate the effect of GSH on redox signalling pathways, instead of being subjected to ES, cells could be challenged with H_2O_2. This partially mimics the redox stress placed upon cells during and following contraction, without the activation of pathways by exercise. This methodology would also enable the H_2O_2 levels to be made consistent between experimental groups, reducing variability in comparison to the current study. Further investigation is required in order to clarify the specific role of GSH depletion, along with an increase in H2O2, without the influence of other pathways activated by contraction. This will allow for a greater understanding of how these two factors affect the expression of the genes of interest.

7. Conclusion

GSH depletion did not interfere with gene expression following myotube electrical stimulation and contraction. Increases in the expression of PPARα and γ, PFK, SOD1, and catalase were seen at baseline, suggesting an effect on glucose metabolism, fatty acid metabolism, and antioxidant potential. These alterations may be due to acute changes in ROS signalling. Further investigation into the specific mechanisms of these effects is required in order to identify other contributing factors such as the MAPK, AMPK, CaMK and NF-κB signalling pathways which are activated by contraction, can be modulated by ROS and/or glutathionylation and are known to control gene expression following exercise.

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Appendix

Mouse primers used for RT-PCR

	Sense (forward) Primer	**Antisense (reverse) Primer**
Catalase	5’-AGCGACCAGATGAAGCAGTG-3’	5’-TCCGCTCTCTGTCAAAGTGTG-3’
CPT-1B	5’-GCACACCAGGCAGTAGCTTT-3’	5’-CAGGAGTTGATTCCAGACAGGTA-3’
CS	5’-TGCCCACACAAGCCATTTG-3’	5’-CTGACACGTCTTTGCCAACTT-3’
GLUT4	5’-GTTGGTCTCGGTGCTCTTAGT-3’	5’-ATAGCATCCGCAACATACTGG-3’
GPx1	5’-CTACACCGAGATGAACGATCTG-3’	5’-TCCGAACTGATTGCACGGG-3’
HK2	5’-GGGCATGAAGGGCGTGTCCC-3’	5’-TCTTCACCCTCGCAGCCGGA-3’
HPRT1	5’-AGTCCCAGCGTCGTGATTAG-3’	5’-TTTCCAAATCCTCGGCATAATGA-3’
NRF1	5’-CCCCCGAGGACACTTCTTATGATG-3’	5’-GGCCGTTTCCGTTTCTTCCCTGTT-3’
NRF2	5’-GGCACAGTGCTCCTATGCGTG-3’	5’-CCAGCTCGACAATGTTCTCCAGC-3’
PFK	5’-GCCACGGTTTCCAATAACGT-3’	5’-AAGTCGTGCAGATGGTGTTCAG-3’
PPARα	5’-GCCTGTCTGTCGGGATGT-3’	5’-GGCTTCGTGGATTCTCTTG-3’
PPARγ	5’-CAAGAATACCAAAGTGCGATC-3’	5’-GAGCTGGGTCTTTTCAGAATAATAAG-3’
SOD1	5’-CCAGTGCAGGACCTCATTTTAAT-3’	5’-TCTCCAACATGCCTCTCTTCATC-3’
SOD2	5’-GCCCCCTGAGTTGTTGAATA-3’	5’-AGACAGGCAAGGCTCTACCA-3’
Tfam	5’-CTGATGGGTATGGAGAAGGAGG-3’	5’-CCAACTTCAGCCATCTGCTCTTC-3’
PGC-1α	5’-TGATGTGAATGACTTGGATACAGACA-3’	5’-GCTCATTGTTGTACTGGTTGGATATG-3’
VEGF-A	5’-GGAGATCCTTCGAGGAGCACTT-3’	5’-GGCGATTTAGCAGCAGATATAAGAA-3’