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Redox remodelling in diaphragm muscle adaptation to chronic sustained hypoxia

Philip Lewis BSc
Department of Physiology

Thesis submitted to National University of Ireland, University College Cork for the award of Doctor of Philosophy

Under the supervision of:
Professor Ken D. O’Halloran (Head of Department)

July 2014
Table of Contents

Declaration ........................................................................................................................................... ii

List of Figures ..................................................................................................................................... iii

List of Tables ....................................................................................................................................... viii

List of Abbreviations ............................................................................................................................. ix

Acknowledgments .................................................................................................................................... xii

Abstract ............................................................................................................................................... xiii

Chapter 1: Introduction ...................................................................................................................... 1

1.1 Hypoxia ........................................................................................................................................... 2

1.2 Skeletal Muscle .............................................................................................................................. 3

1.2.1 The Respiratory Muscles ........................................................................................................... 4

1.2.2 The Diaphragm ............................................................................................................................ 5

1.2.3 The Sternohyoid ............................................................................................................................ 5

1.2.4 Hind-limb muscles ....................................................................................................................... 6

1.3 Clinical Relevance .......................................................................................................................... 6

1.3.1 Chronic Lung Diseases ............................................................................................................... 7

1.3.2 Obstructive Sleep Apnoea .......................................................................................................... 7

1.4 Mechanism of Muscle Contraction .................................................................................................. 8

1.5 Functional Adaptations in Hypoxia ................................................................................................. 9

1.6 Molecular Adaptations in Hypoxia ................................................................................................. 12

1.6.1 Structural Adaptations ............................................................................................................... 12

1.6.2 Metabolic Adaptations ................................................................................................................. 15

1.6.3 Calcium Handling ....................................................................................................................... 19

1.6.4 The Sarcomere ............................................................................................................................. 19

1.6.5 Myokines ..................................................................................................................................... 20

1.7 Redox Homeostasis ....................................................................................................................... 20
Chapter 2: Methods ................................................................. 29
2.1 Animal Model ................................................................. 30
  2.1.1 Sample Size Calculation ............................................... 30
  2.1.2 Groups ....................................................................... 31
  2.1.3 Tempol and NAC .......................................................... 33
2.2 Muscle Tissue Preparation .................................................. 33
2.3 Redox Proteomics ............................................................. 34
  2.3.1 Total Protein Carbonyl and Free Thiol Content ................. 34
  2.3.2 2D Redox Proteomics .................................................... 34
  2.3.3 Image Analysis ............................................................. 35
  2.3.4 Protein Digestion and Identification ................................ 35
  2.3.5 Statistical Analysis ....................................................... 36
2.4 Spectrophotometry ............................................................ 36
  2.4.1 Antioxidants ............................................................... 39
    2.4.1.1 Catalase Activity ................................................... 39
    2.4.1.2 Total Glutathione Content ...................................... 39
  2.4.2 Metabolic Enzymes ....................................................... 41
    2.4.2.1 Glucose-6-Phosphate Dehydrogenase Activity ............. 41
    2.4.2.2 Fructose-1, 6-bisphosphate Aldolase A Activity .......... 41
    2.4.2.3 Glycerol-3-Phosphate Dehydrogenase Activity .......... 42
    2.4.2.4 Glyceraldehyde-3-phosphate Dehydrogenase Activity .... 43
    2.4.2.5 Lactate Dehydrogenase Activity .............................. 44
    2.4.2.6 Aconitase Activity ............................................... 44
2.4.2.7 Creatine Kinase Activity ................................................................. 45
2.4.3 Hypertrophy/Atrophy Signalling .......................................................... 46
  2.4.3.1 Chymotrypsin-Like Proteasome Activity ............................................. 46
  2.4.3.2 Phospho-FOXO3a (Thr32) content ................................................... 46
  2.4.3.3 mTOR and phospho-mTOR (Ser2448) content .................................... 47
  2.4.3.4 Phospho-Akt, phospho-p70S6K, phospho-GSK-3β, phospho-S6RP content ................................................................. 47
  2.4.3.5 Phospho-ERK 1/2, phospho-p38, phospho-JNK content ......................... 48
  2.4.4 HIF-1α content ................................................................................... 48
  2.4.5 Statistical Analysis .............................................................................. 49
2.5 Isotonic Muscle Function ............................................................................ 49
  2.5.1 Ex Vivo Muscle Preparation .................................................................. 49
  2.5.2 Protocol .................................................................................................. 50
  2.5.3 Data Analysis ........................................................................................ 50
2.6 Succinate Dehydrogenase Histochemistry Analysis ...................................... 51

Chapter 3: Redox remodelling in diaphragm muscle following chronic sustained hypoxia ................................................................................................................. 53
3.1 Chapter Introduction .................................................................................... 54
3.2 Chapter Results ........................................................................................... 56
  3.2.1 Section A: Total Protein Carbonyl and Free Thiol Content ...................... 56
    3.2.1.1 Diaphragm ....................................................................................... 58
    3.2.1.2 Sternohyoid ................................................................................... 58
    3.2.1.3 EDL ............................................................................................... 61
    3.2.1.4 Soleus ............................................................................................. 61
    3.2.1.5 Total Protein Carbonyl and Free Thiol Content expressed as percentage of control ................................................................. 64
  3.2.2 Section B: Redox Proteomics .................................................................. 67
### Chapter 4: Metabolic adaptation in the diaphragm following chronic sustained hypoxia

4.1 Chapter Introduction ................................................................. 83
4.2 Chapter Results .............................................................................. 87
   4.2.1 Section A: SDH activity at the fibre level ................................ 87
   4.2.2 Section B: Metabolic enzyme activities and HIF-1α ............ 91
4.3 Chapter Discussion ........................................................................ 102
   4.3.1 SDH activity at the fibre level ................................................. 102
   4.3.2 Metabolic enzyme activities and HIF-1α .................... 103
   4.3.3 Conclusion .......................................................................... 106

### Chapter 5: Atrophy in the diaphragm after chronic sustained hypoxia

5.1 Chapter Introduction .................................................................... 109
5.2 Chapter Results .............................................................................. 114
   5.2.1 Section A: Chymotrypsin-like Proteasome Activity ......... 114
   5.2.2 Section B: Akt signalling cascade ........................................ 116
   5.2.3 Section C: MAP Kinases ........................................................ 121
5.3 Chapter Discussion ........................................................................ 125
   5.3.1 Chymotrypsin-like Proteasome Activity .............................. 125
   5.3.2 Akt signalling cascade ......................................................... 126

---

3.2.3 Section C: Antioxidants .................................................................. 71
3.3 Chapter Discussion ............................................................................ 72
   3.3.1 Total Protein Carbonyl and Free Thiol Content ................... 72
   3.3.2 Redox Proteomics ................................................................. 74
   3.3.3 Antioxidants ........................................................................ 80
   3.3.4 Conclusion .......................................................................... 81

---

**Chapter 4: Metabolic adaptation in the diaphragm following chronic sustained hypoxia** ................................................................. 82

4.1 Chapter Introduction .................................................................... 83
4.2 Chapter Results .............................................................................. 87
   4.2.1 Section A: SDH activity at the fibre level ................................ 87
   4.2.2 Section B: Metabolic enzyme activities and HIF-1α ............ 91
4.3 Chapter Discussion ........................................................................ 102
   4.3.1 SDH activity at the fibre level ................................................. 102
   4.3.2 Metabolic enzyme activities and HIF-1α .................... 103
   4.3.3 Conclusion .......................................................................... 106

**Chapter 5: Atrophy in the diaphragm after chronic sustained hypoxia** .................................................. 108

5.1 Chapter Introduction .................................................................... 109
5.2 Chapter Results .............................................................................. 114
   5.2.1 Section A: Chymotrypsin-like Proteasome Activity ......... 114
   5.2.2 Section B: Akt signalling cascade ........................................ 116
   5.2.3 Section C: MAP Kinases ........................................................ 121
5.3 Chapter Discussion ........................................................................ 125
   5.3.1 Chymotrypsin-like Proteasome Activity .............................. 125
   5.3.2 Akt signalling cascade ......................................................... 126
Declaration

I declare that this thesis is not under consideration for other qualifications in University College Cork or elsewhere and that the work contained within is original and my own.

Signed: ________________

Philip Lewis

July 2014
List of Figures

Chapter 1: Introduction

Figure 1.1: Superoxide and nitric oxide pathways of protein oxidation and antioxidant-induced reduction

Figure 1.2: Generators of reactive species in the skeletal muscle cell

Figure 1.3: Hypothesised and experimentally demonstrated relationship between isometric force of skeletal muscle and cellular redox state

Chapter 2: Method

Figure 2.1: Oxycycler set-up and chamber oxygen profiles for treatment period

Figure 2.2: Workflow and principles of spectrophotometry

Figure 2.3: Decomposition of hydrogen peroxide by the enzyme catalase

Figure 2.4: Recycling mechanism of total glutathione assay

Figure 2.5: G6PD catalyses the reversible oxidation of D-glucose-6-phosphate into 6-phospho-D-glucono-1,5-lactone

Figure 2.6: Aldolase catalyses the reversible reduction of fructose-1-6-bisphosphate into both 3-phosphoglyceraldehyde and dihydroxyacetone phosphate

Figure 2.7: G3PD catalyzes the reversible reduction of dihydroxyacetone phosphate into glycerol-3-phosphate

Figure 2.8: GAPDH catalyzes the reversible reduction of glyceraldehyde-3-phosphate into D-glycerate-1,3-bisphosphate

Figure 2.9: LDH catalyzes the reversible reduction of pyruvate into lactate

Figure 2.10: Aconitase catalyzes the reversible reduction of citrate into isocitrate

Figure 2.11: Creatine Kinase catalyzes the reversible phosphorylation of creatine into phosphocreatine

Figure 2.12: Representation of method of protein content determination using the ‘Mesoscale’ assays

Chapter 3: Protein Redox Remodelling

Figure 3.1: Representative images of 1D gel protein separations
Figure 3.2: Diaphragm protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 3.3: Sternohyoid protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 3.4: EDL protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 3.5: Soleus protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 3.6: Skeletal muscle protein carbonyl content expressed as a percentage of control after one, three, and six weeks of chronic sustained hypoxia

Figure 3.7: Skeletal muscle protein free thiol content expressed as a percentage of control after one, three, and six weeks of chronic sustained hypoxia

Figure 3.8: Representative image of 2D-PAGE protein profiles after Coomassie staining of proteins

Figure 3.9: Diaphragm and Sternohyoid total GSH content after six weeks of sustained hypoxia compared to normoxic control

Figure 3.10: Diaphragm catalase activity after one, three, and six weeks of sustained hypoxia compared to control

Figure 3.11: In-vitro motility assay for control and oxidised (H₂O₂ treated) F-actin (unpublished pilot study data from Persson, M., 2013 and shown here with permission)

Chapter 4: Metabolic Enzyme Activity

Figure 4.1: Overview of metabolism pathways in skeletal muscle

Figure 4.2: 10μm transverse section of rat diaphragm and sternohyoid muscle stained for SDH activity

Figure 4.3: Representative area-density plots for diaphragm and sternohyoid

Figure 4.4: Scatterplots of cluster centroids for diaphragm and sternohyoid in CH-treated and normoxic rats

Figure 4.5: Diaphragm aconitase and creatine kinase activity after one, three, and six weeks of sustained hypoxia compared to normoxic controls

Figure 4.6: Diaphragm aldolase and G3PD activity after one, three, and six weeks of sustained hypoxia compared to normoxic controls
Figure 4.7: Diaphragm G6PD activity and LDH activity after one, three, and six weeks of sustained hypoxia compared to normoxic controls

Figure 4.8: Diaphragm and sternohyoid GAPDH activity after one and three weeks of sustained hypoxia compared to normoxic controls

Figure 4.9: EDL and soleus GAPDH activity after one and three weeks of sustained hypoxia compared to normoxic controls

Figure 4.10: Sternohyoid, EDL and soleus LDH activity after one and three weeks of sustained hypoxia compared to normoxic controls

Figure 4.11: Diaphragm and sternohyoid HIF1-α content after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 4.12: Achieving hypoxia tolerance as described by Ramirez et al., (2007)

Chapter 5: Atrophy Mechanism and Signalling

Figure 5.1: Hypertrophy/Atrophy signalling in muscle

Figure 5.2: Chymotrypsin-like proteasome activity in diaphragm, sternohyoid, EDL, and soleus after sustained hypoxia compared to normoxic controls

Figure 5.3: Diaphragm phospho-mTOR and phospho-FOXO3a content after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 5.4: Diaphragm phospho-Akt and phospho-GSK-3β content after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 5.5: Diaphragm phospho-p70S6K and phospho-S6RP content after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 5.6: Diaphragm phospho-MAPK contents after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 5.7: Sternohyoid phospho-MAPK contents after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Chapter 6: Diaphragm Isotonic Function ± Chronic Antioxidant Supplementation

Figure 6.1: Diaphragm isometric twitch kinetics after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.2: Diaphragm peak specific twitch force and tetanic force after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia
Figure 6.3: Diaphragm peak specific shortening velocity and shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.4: Diaphragm peak specific power and work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.5: Diaphragm shortening velocity-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.6: Diaphragm shortening-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.7: Diaphragm work-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.8: Diaphragm power-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.9: Diaphragm Fatigue Tolerance: Shortening velocity after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.10: Diaphragm Fatigue Tolerance: Shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.11: Diaphragm Fatigue Tolerance: Work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.12: Diaphragm Fatigue Tolerance: Power after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.13: Sternohyoid isometric twitch kinetics after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.14: Sternohyoid peak specific twitch force and tetanic after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.15: Sternohyoid peak specific shortening velocity and shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.16: Sternohyoid peak specific power and work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.17: Sternohyoid shortening velocity-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.18: Sternohyoid shortening-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia
Figure 6.19: Sternohyoid work-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.20: Sternohyoid power-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.21: Sternohyoid Fatigue Tolerance: Shortening velocity after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.22: Sternohyoid Fatigue Tolerance: Shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.23: Sternohyoid Fatigue Tolerance: Work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 6.24: Sternohyoid Fatigue Tolerance: Power after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Appendix 1:

Figure 8.1: C57Bl6/J percentage haematocrit after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 8.2: C57Bl6/J body weights after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 8.3: C57Bl6/J right ventricle/left ventricle (+ septum) weights after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 8.4: C57Bl6/J right ventricle/body weights after one, three and six weeks of sustained hypoxia compared to normoxic controls

Figure 8.5: C57Bl6/J percentage haematocrit after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 8.6: C57Bl6/J right ventricle/left ventricle (+ septum) weights after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 8.7: C57Bl6/J right ventricle/body weights after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 8.8: C57Bl6/J body weights after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia

Figure 8.9: C57Bl6/J water consumption after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia
List of Tables

Chapter 3: Protein Redox Remodelling

Table 3.1: Identification of proteins undergoing significant redox remodelling in the diaphragm muscle after six weeks of sustained hypoxia

Table 3.2: Identification of proteins undergoing significant redox remodelling in the sternohyoid muscle after six weeks of sustained hypoxia
List of Abbreviations

A  absorbance
Aldolase  fructose bis-phosphate aldolase A
AMP(K)  adenosine monophosphate (kinase)
ANOVA  analysis of variance
ASK  apoptosis stimulating kinase
BCA  bicinchoninic assay
BNIP-3  BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CA  carbonic anhydrase
CaM  calmodulin
CaMKII  calmodulin kinase II
CH  chronic sustained hypoxia
CK  creatine kinase
COPD  chronic obstructive pulmonary disease
DHPR  dihydropyridine receptor
DTNB  5-5-dithio-bis-2-nitroitrobenzoic acid
DTT  dithiothreitol
E-C  excitation-contraction
EDL  extensor digitorum longus
ERK  extracellular signal related kinase
ETC  electron transport chain
FABP  fatty acid binding protein
FAD/H₂  flavin adenine dinucleotide/reduced
FCSA  fibre cross sectional area
F-F  force-frequency relationship
F₂O₂  fraction of inspired oxygen
Fmax  peak specific tetanic force
FOXO  forkhead box (transcription factor)
FTSC  fluorescein-thiosemicarbazon
G3PD  glycerol-3-phosphate dehydrogenase
G6PD  glucose-6-phosphate dehydrogenase
GAPDH  glyceraldehyde-6-phosphate dehydrogenase
GPx glutathione peroxidase
GR glutathione reductase
(t)GSH (total) glutathione
GSSG oxidised glutathione/glutathione disulphide
GSK glycogen synthase kinase
Hgb haemoglobin
HIF hypoxia inducible factor
HSP heat-shock protein
IAF iodoacetamido-fluorescein
IEF isoelectric focusing
IGF insulin-like growth factor
JNK C-Jun-N-terminal kinase
Lo optimal length
L/Lo length/optimal length
LDH lactate dehydrogenase
MAPK mitogen activated protein kinases
mATPase myosin ATPase
MHC myosin heavy chain
MS mass spectrometry
mTOR(C) mammalian target of rapamycin (complex)
NAC n-acetyl-cysteine
NAD(P)/H nicotinamide dinucleotide (phosphate)/reduced
NFAT nuclear factor of activated T-cells
NHE sodium/hydrogen exchanger
NO nitric oxide
NOS nitric oxide synthase
NOX NADPH oxidase
OSA obstructive sleep apnoea
p70S6K phospho-protein 70 ribosome S6 kinase
PAGE polyacrylamide gel electrophoresis
PaO_2 partial pressure of oxygen in arterial blood
PGC1-α peroxisome proliferator-activated receptor γ co-activator 1-α
phospho-R phosphorylated-protein
P_i inorganic phosphate
PI3K phosphatidylinositol 3 kinase
Pmax peak specific power
PMSF phenylmethylsulfonyl fluoride
PO_2 partial pressure of oxygen
PPARγ peroxisome proliferator-activated receptor γ
RCR respiratory control ration
RIPA radio-immunoprecipitation assay
RLU relative luminescence units
ROS reactive oxygen species
RyR ryanodine receptor
S6RP ribosomal protein S6
SDH succinate dehydrogenase
SDS sodium dodecyl sulfate
SEM standard error of the mean
SERCA sarco/endoplasmic reticulum Ca^{2+} ATPase
Smax peak specific shortening
SOD superoxide dismutase
SR sarcoplasmic reticulum
T50 peak twitch tension half relaxation time
TCA trichloroacetic acid
TCA cycle tricarboxylic acid cycle
Tn troponin
TTP time to peak twitch tension
UCP uncoupling protein
Wmax peak specific work
XO xanthine oxidase
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Abstract

Introduction: Chronic sustained hypoxia (CH) induces functional weakness, atrophy, and mitochondrial remodelling in the diaphragm muscle. Animal models of CH present with changes similar to patients with respiratory-related disease. Elucidating the molecular mechanisms that drive diaphragm adaptations to CH is clinically important. Reactive oxygen species (ROS) are produced by – and contribute to – damaged mitochondria while hypoxia and increased muscle contractile activity also promote ROS formation. We hypothesize that ROS are pivotal in diaphragm muscle adaptation to CH.

Method: C57BL6/J mice were exposed to CH (FiO₂=0.1) for one, three, and six weeks. Sternohyoid (upper airway dilator), extensor digitorum longus (EDL), and soleus were studied as reference muscles as well as the diaphragm which was the major focus of this thesis. The diaphragm was profiled using a redox proteomics approach followed by mass spectrometry. Following this, redox-modified metabolic enzyme activities and atrophy signalling were assessed using spectrophotometric assays and ELISA. Diaphragm isotonic performance was assessed after six weeks of CH ± chronic antioxidant supplementation.

Results: Protein carbonyl and free thiol content in the diaphragm were increased and decreased respectively after six weeks of CH – indicative of protein oxidation. These changes were temporally modulated and muscle specific. Extensive remodelling of metabolic proteins occurred and the stress reached the cross-bridge. Metabolic enzyme activities in the diaphragm were, for the most part, decreased by CH and differential muscle responses were observed. Redox sensitive chymotrypsin-like proteasome activity of the diaphragm was increased and atrophy signalling was observed through decreased phospho-FOXO3a and phospho-mTOR. Phospho-p38 MAPK content was increased and this was attenuated by antioxidant treatment. Hypoxia decreased power generating capacity of the diaphragm and this was restored by N-acetyl-cysteine (NAC) but not by tempol.
Conclusion: Redox remodelling is pivotal for diaphragm adaptation to chronic sustained hypoxia. Muscle changes are dependent on duration of the hypoxia stimulus, activity profile of the muscle, and molecular composition of the muscle. The working respiratory muscles and slow oxidative fibres are particularly susceptible. Atrophy signalling through FOXO3a is likely mediated by p38MAPK and not Akt. NAC (antioxidant) may be useful as an adjunct therapy in respiratory-related diseases characterised by hypoxic stress.
Chapter 1: Introduction
1.1 Hypoxia

Hypoxia is a condition of insufficient O$_2$ delivery to – or utilisation by – the body’s cells and tissues such that metabolic demands are not adequately met. An abrupt or rapid depletion in available oxygen (acute hypoxia) may result from exercise, asphyxia, airway obstruction or acute haemorrhaging. In comparison, chronic sustained hypoxia (CH) is encountered in chronic respiratory disease or upon ascent to high altitude – although there may be overlap between chronic and acute under some circumstances such as exacerbations of disease. Hypoxia is also featured during embryonic development, ischaemia and tumours. Of course these generally present focal hypoxia as opposed to widespread hypoxaemia (low blood oxygen content/partial pressure). Approximately 90-100mmHg constitutes the normal range of partial pressure of oxygen in arterial blood (PaO$_2$). Clinically, the PaO$_2$ in chronic obstructive pulmonary disease (COPD) patients can drop below 60mmHg [1,2]. PaO$_2$ has been reported as low as 28mmHg near the summit of Mt Everest [3].

Hypoxia is classified into five distinct types: hypoxic, anaemic, stagnant, histotoxic, and genetic. Hypoxic hypoxia is attributable to insufficient O$_2$ in inspired air, decreased oxygen partial pressure (PO$_2$) in the alveolus or inadequate O$_2$ transfer from the alveoli into pulmonary capillaries. It may transpire by means of ascent to high altitude, hypoventilation, diffusion abnormalities, pulmonary shunting or ventilation/perfusion mismatch. Anaemic hypoxia is an abnormally low O$_2$ carrying capacity of the blood due to either a lack of haemoglobin (Hgb) (e.g. during hypovolaemia) or poor Hgb-oxygen binding capacity (e.g. carbon monoxide poisoning). Stagnant hypoxia is a result of poor blood perfusion such as occurs in several cardiovascular pathologies. Histotoxic hypoxia arises when tissues cannot metabolise sufficient oxygen despite adequate PO$_2$ and oxygen delivery (e.g. sepsis). Genetic hypoxia can arise from mutations in genes responsible for degradation of hypoxia-responsive proteins in normoxic conditions (e.g. congenital polycythaemia) and can lead to dysfunction of oxygen utilisation and homeostasis in cells.

The physiological response to hypoxia depends on the rate and degree of exposure e.g. a mountain climber who has spent some weeks at high altitude is able to remain
conscious and continue on his upward route whereas a pilot in an unpressurised aircraft at the height of the summit of Mt. Everest (8848m) who loses his oxygen supply would lose consciousness in a matter of minutes [4]. Classic hypoxia acclimatisation responses include reflex hyperventilation followed by progressive decline before ventilation plateaus at a higher level than in normoxia. Increases in [Hgb], capillary density and heart rate also occur. These changes may be considered beneficial but CH can result in pathologies such as hypoxic pulmonary vasoconstriction, pulmonary hypertension, and cor pulmonale. These result from vascular wall remodelling including thickening of vessel walls and extension of smooth muscle cells into previously non-muscular arterioles. Elevated pulmonary arterial pressures result in increased right heart workload which can lead to right heart failure. Polycythaemia is considered contributory to Monge’s disease in Andean populations and elevated blood viscosity can lead to left heart problems. High altitude is defined as >2,500m above sea level and full acclimatisation can take up to three weeks or more [5].

1.2 Skeletal Muscle

Skeletal muscle, as the name suggests, is connected at one or both ends to the skeleton and is the main effector organ of the locomotor system with an attendant role in thermoregulation, excretory functions, and more recently a role in endocrine physiology. It consists of bundles of long, multinucleated, thick or thin, fused fibres that appear transversely striated under a microscope. Muscle fibres differ in their abilities to generate power and carry out mechanical work thus resulting in the heterogeneity observed in skeletal muscles. Thin, slow, oxidative fibres are designed for endurance activities such as postural control whereas thick, fast, glycolytic fibres are required where more powerful contractions against heavy loads are necessary. There are several classifications of fibre type – the most common classifications being by myosin heavy chain (MHC) isoform composition (types I, IIa, IIx and IIb; slow to fast respectively) or by kinetics and metabolic activities (slow oxidative, fast oxidative and fast glycolytic). Intermediate fibre types also occur and fibre type classification may vary with the technical approach used. MHC isoforms are the main determinant of contractile properties; the most abundant protein in skeletal
muscle; and several methods may be used to identify MHC isoform composition including immunofluorescent imaging and polyacrylamide gel electrophoresis (PAGE). Muscle fibre type composition is regulated by gene expression and microarray studies have revealed significant gene expression differences in fast and slow muscles [6]. At the protein level, mitogen activated protein kinase (MAPK) pathways, calcineurin, calmodulin (CaM) kinase IV, peroxisome proliferator γ co-activator 1-α (PGC1-α) and hypoxia-inducible factor (HIF) transcription factors are involved [7–15] in regulating fibre type composition. HIF-1α and HIF-2α transcription factors are hypoxia-responsive and considered regulatory in fast and slow muscle fibre differentiation respectively [13–15].

Ultimately, the molecular compositions of slow and fast fibre types are heterogeneous and thus respond differently to different stimuli. Furthermore, skeletal muscle is an extremely malleable tissue. Hypertrophy/atrophy, metabolic activity and molecular composition changes and even fibre-type switching can occur as a result of training and/or disuse [16–21]. This becomes obvious when comparing professional weight lifters with long distance runners and sedentary individuals. Hypoxia, both short and long term exposures of different paradigms, induces differential muscle remodelling [22–27], ostensibly linked to molecular composition and functional role of the muscle. The sum total of skeletal muscle in the human body consumes approximately 20% of available oxygen and more than this during exercise. The effects of CH exposure on skeletal muscle and the molecular mechanisms that drive adaptation to CH, specifically in the diaphragm muscle for reasons that will become clear in the following paragraphs, will be explored in this thesis.

1.2.1 The Respiratory Muscles
The respiratory muscles, or muscles of breathing, are unique as continuous rhythmic activity without rest is required to sustain human life. Despite this, they have the same general structure and operational manner as limb muscles. The respiratory muscles encompass the diaphragm, the extrinsic and intrinsic intercostals and approximately 20 upper airway skeletal muscles.
1.2.2 The Diaphragm

The diaphragm, the primary inspiratory pump muscle critical for respiration, is composed of a mixture of fibre types [22], is present in some form in more than 5000 mammalian species, and is almost a completely unique trait of mammals [28]. Diaphragmatic contraction increases thoracic volume and subsequent negative pressure in the airways allows air at atmospheric pressure to flow from the external environment into the lungs. Upon relaxation, air can be drawn out passively by elastic recoil. Both force generation and fatigue resistance are important functional parameters for the diaphragm given its unique anatomical shape, continuous rhythmic activity (even during sleep), and its requirement to produce faster contractions such as during tachypnoea. This is reflected by its mixed fibre type distribution. Passive, involuntary breathing uses slow fibre types while specific recruitment of fast fibres occurs in the diaphragm of heat-induced rapidly ventilating rabbits [29]. The diaphragm is a dome-shaped membrane separating the thoracic and the abdominal cavities. It is innervated exclusively from the phrenic nerves originating from C3, C4 and C5 which provide both motor and sensory control. It is under both voluntary and involuntary control from the central nervous system. In addition to breathing, the diaphragm has physiological roles in excretion through increasing intra-abdominal pressure and acid reflux control through exerting pressure on the lower oesophageal sphincter. The major arterial blood supply comes from the left and right phrenic arteries. In hypoxia, blood flow is diverted away from several vascular beds to the diaphragm but not to other skeletal muscles [30–32], thus highlighting the importance of the diaphragm in hypoxic conditions.

We are also interested in other respiratory and limb muscles from a comparative point of view and so the other muscles studied in this thesis alongside the diaphragm, and their functional roles, are described below.

1.2.3 The Sternohyoid

Pharyngeal dilator muscles regulate upper airway patency during breathing [33]. As the pharyngeal airway is not supported by bone or cartilage, it would collapse with the negative pressures associated with the initial phase of inspiration were it not for the activity of these muscles [34]. The pharyngeal dilator muscles provide much
needed support to the structure by contracting synchronously with the diaphragm [35]. The transition from wakefulness to sleep is associated with decrements in pharyngeal muscle tone, thus functional changes in these muscles will be of physiological consequence [33,36–39]. The sternohyoid, a representative upper airway dilator muscle which we study in parallel to the diaphragm, is composed almost entirely of fast type IIb fibres in rodents [22] (note: there is no information available regarding the composition of this muscle in humans). Sternohyoid muscle length has been shown to be inversely proportional to pharyngeal airway volume [40].

1.2.4 Hind-limb muscles
The extensor digitorum longus (EDL) and soleus hind-limb muscles are useful for study alongside the diaphragm for consideration of muscle adaptations resulting from muscle specificity or related to the molecular composition or activity profile of a given muscle under a particular stimulus – CH in this case. The EDL is a powerful, fast glycolytic muscle, located along the anterolateral side of the leg and deep to the tibialis anterior, whose function involves dorsiflexion of the foot. It is composed predominantly of fast glycolytic fibres. In comparison, the soleus is a highly enduring muscle located deep to the gastrocnemius in the posterior compartment of the leg, composed of a very high proportion of slow oxidative fibres, and plays an important functional role in postural maintenance.

1.3 Clinical Relevance
Respiratory muscles increase their workload against hypoxic insult through reflex hyperventilation. The respiratory frequency of CH rats is 30–40% greater than normoxic controls [41]. In contrast, there may be deconditioning of limb muscles due to reduced activity [42]. Muscle remodelling is a feature of high altitude and respiratory-related diseases where hypoxia is featured [43–46], but the role of CH in these processes is not clear at present.
1.3.1 Chronic Lung Diseases
Asthma, bronchopulmonary dysplasia, and COPD (COPD is an umbrella term for emphysema and bronchitis) constitute chronic lung diseases. These lung diseases perturb breathing and are progressive. COPD alone is the 12th leading cause of death worldwide, 5th leading cause in Western countries and, according to reports by the World Health Organisation, is predicted to climb to the 3rd leading global cause of death by 2030. The main symptoms include sputum production, chronic and sometimes severe coughing, and shortness of breath. Co-conditions and morbidities can include hypertension, ischaemic heart disease, diabetes, muscle wasting and malaise. The primary cause of COPD is tobacco smoking but there are genetic and environmental risk factors. There is no cure. Respiratory muscle dysfunction is implicated in the pathogenesis and/or progression of COPD. Maximum inspiratory pressure is correlated with survival in COPD patients [47–49], thus functional remodelling in the diaphragm muscle is clinically relevant. Furthermore, the COPD diaphragm is more susceptible to muscle injury after inspiratory loading [50].

1.3.2 Obstructive Sleep Apnoea
Obstructive sleep apnoea (OSA) is a condition of sleep-disordered breathing, characterised by repetitive airway collapse, increased airway resistance, acute intermittent periods of hypoxia and is associated with upper airway muscle dysfunction that is thought to contribute to its progression [36,37,51,52]. It is known to affect 9-24% of the population although it is considered that this number may be significantly larger with many patients undiagnosed. Prevalence is increasing with obesity recognised as a significant risk factor. Comorbidities include obesity, hypertension and cognitive impairments. OSA in conjunction with COPD (overlap syndrome) is more common than OSA alone [53]. Nocturnal oxygen desaturation develops in COPD independent of apnoea and is more severe in the overlap syndrome [54].

COPD and OSA represent two of the most prevalent chronic respiratory diseases [55]. Elucidating molecular mechanisms that underpin respiratory muscle adaptation to CH will be important in shaping new therapeutic strategies in diseases where hypoxia and/or muscle dysfunction are featured. This is especially important given
that exercise programmes are increasingly being considered as adjunct-therapies in respiratory disease. There is also interest in mechanisms controlling respiratory muscle performance and adaptation to hypoxia from the fields of exercise physiology, high altitude physiology and aviation.

We cannot consummately say that COPD-induced functional adaptations in respiratory muscle are a direct result of hypoxia and/or reflex hyperventilation as there may be other disease-related confounding influences (e.g. hypercapnia and inflammation). However, the observed functional adaptations are very similar to adaptations observed in animal models of CH. COPD patients have a lower trans-diaphragmatic pressure generating capacity as well as improved fatigue tolerance in their diaphragm muscle [43,44,56,57] or at least, fatigue tolerance is not reduced despite them being at a mechanical disadvantage [45]. Hypoxia-induced functional adaptations in respiratory muscles are discussed below in section 1.5.

1.4 Mechanism of Muscle Contraction

The stimulus for muscle activity are neural impulses. Muscle fibres themselves are excitable cells. At the neuromuscular junction, electrical impulses from neurons are amplified chemically to raise the motor endplate voltage above threshold and initiate excitation-contraction (E-C) coupling. Upon reaching threshold voltage, Na$^+$ channels, abundant in the sarcolemma, spread the electrical signal across the muscle and down through invaginations called transverse-tubules which run perpendicular to the sarcolemma. These t-tubules carry the signal to voltage ‘sensors’ that transduce and transmit the signal to the structures responsible for contraction. Dihydropyridine receptors (DHPRs) concentrated in the t-tubules undergo a voltage-dependent conformational change resulting in their interaction with ryanodine receptors (RyRs) of the sarcoplasmic reticulum (SR – intracellular Ca$^{2+}$ store). The RyRs are Ca$^{2+}$ release channels whose pores open with DHPR interaction allowing Ca$^{2+}$ to flood down its concentration gradient into the myoplasm. Ca$^{2+}$, and ATP from metabolism, are the cofactors required for sarcomere shortening [58,59].
The Sarcomere and Sliding-Filament Theory

The sarcomere is the basic unit of muscle function and is composed of thick myosin and thin actin filaments arranged in parallel. In 1954, using high resolution microscopy to visualise the action of actin and myosin, Huxley, Hanson, and Niedergerke proposed the sliding-filament theory of muscle contraction [60,61]. They describe the action of actin, which is tethered to protein (termed the ‘Z’ disc) at the lateral end of each sarcomere, sliding past stationary myosin at various stages of contraction. As actin moves, it pulls the Z-discs closer together resulting in sarcomere, and thus muscle, shortening. This theory has since been refined after the observation that myosin ‘heads’ (globular domains at the ends of filamentous myosin) can bind to actin, forming a cross-bridge, and pull upon the actin to shorten the sarcomere [62].

Cross-bridge Cycling

In a resting sarcomere, the tropomyosin protein blocks the myosin head binding sites on actin. Ca$^{2+}$, introduced from the SR, can bind to troponin which is attached to tropomyosin and cause a conformational change in the tropomyosin that in turn exposes the myosin head binding sites [58,63]. ATP which has bound to myosin is hydrolysed by the myosin-ATPase (mATPase) enzyme into ADP and inorganic phosphate (P$_i$). The energy released by this reaction activates the myosin head into its extended position where it binds to exposed binding sites on actin. Myosin releases ADP, returning the myosin head to its unextended position. As it returns, it is still attached to actin and pulls the actin filament along (‘the power stroke’). ATP can now bind to myosin which breaks the actin-myosin bond. ATP hydrolysis by mATPase will again facilitate binding if binding sites on actin are available. The collective binding of numerous myosin heads to actin combine to move the actin relative to the myosin filaments.

1.5 Functional Adaptations in Hypoxia

Dissecting the information from ex vivo muscle bath studies is complicated. The role of temperature in skeletal muscle performance assessment ex vivo must be taken into
account. At 37°C metabolic activity is greatest and therefore muscle viability in a tissue bath will diminish faster. There is evidence supporting an increase in $\text{O}_2^-$ production in mammalian skeletal muscle as temperature increases between 32 and 37°C resulting in irreversible force depression [64]. However, at lower temperatures metabolic activity is not occurring at a physiological rate and the complexion of muscle function may be altered somewhat because of this. Clearly, there is a trade-off in reducing *ex vivo* muscle rundown with physiological redox signalling. It is debatable as to what temperature gives more physiological results. Differences in hypoxic intensity, exposure times and different experimental paradigms employed may also account for discrepancies in the literature e.g. reduced air density in hypobaric hypoxia will reduce the work of breathing and may affect rates of diffusion in airways differential to normobaric hypoxia. Moreover, isometric contractions in respiratory muscle, particularly the diaphragm, would not result in thoracic volume and pressure changes which are required for breathing, yet most studies have focussed on isometric properties of the muscle. There has been no study to date on isotonic function in CH animal models. Nevertheless, isometric performance capacities of muscle are still a good indicator of potential *in vivo* performance and serve to link performance changes to molecular changes.

**Twitch Tension and Kinetics**

Peak twitch tension in rat diaphragm after six weeks of CH exposure to 0.1 fraction of inspired $\text{O}_2$ ($F_{\text{i}}\text{O}_2=0.1$; equivalent to 5800-6200m above sea level) has been observed to significantly increase, remain the same and significantly decrease at 25°C [26], 30°C [22] and 37°C [65] respectively. There were no significant changes in contractile kinetics. There was no change in twitch tension or contraction kinetics in rat sternohyoid after six weeks of CH exposure at 30°C [22], however a significant increase in twitch tension was observed at 25°C [26]. Similar temperature-dependent results were seen in EDL and soleus muscles of both rats and mice [65–67].

**Force-frequency relationship**

Four weeks of CH had no effect on the force-frequency relationship (F-F) in mouse diaphragm at 37°C [67]. Six weeks of CH depressed the F-F in rat diaphragm at 37°C [65] and 30°C [22] but enhanced F-F at 25°C [26]. After six weeks of CH,
there was no change to F-F in rat sternohyoid muscle at 30°C [22] or 25°C [26], nor was there any change in the soleus at 37°C, however F-F was depressed in rat EDL at 37°C [65]. Four weeks of CH had no effect on F-F in mouse soleus at 37°C [67].

**Fatigue Tolerance**

Fatigue tolerance in rat diaphragm significantly increased after six weeks of CH at 30°C [22], however it was unchanged at 37°C [65] and 25°C [26]. Four weeks of CH had no effect on fatigue tolerance in mouse diaphragm at 37°C [67]. Six weeks of CH exposure decreased fatigue tolerance in rat sternohyoid at 25°C [26], however no change was observed at 30°C [22]. After four weeks of CH, the post-stimulation relaxation time/peak force in rat soleus was significantly decreased indicating a reduced tolerance to fatigue at 25°C [66] although no change was observed at 37°C [65] with the same results observed in the EDL muscle at 37°C.

**Muscle Performance in Acute Hypoxia**

Acute hypoxia has severe detrimental effects on respiratory muscle function *ex vivo*. Force production, shortening velocity, total shortening and fatigue tolerance are all impaired [68,69].

**Single-Fibre Function**

Single fibre studies on diaphragm and soleus muscle from animals exposed to four weeks of CH reveal significant reductions in force generating capacity of type I and type II fibres from both muscles, with the type II fibres apparently more severely affected [70]. No changes were observed to shortening velocities but power production was significantly decreased.

The general consensus is that CH-induced functional adaptations in the diaphragm appear to maintain/improve performance [67] in comparison to limb muscles. However, force generating capacity in the diaphragm at 35-37°C is compromised and thus there is diaphragm muscle weakness following CH exposure. Unpublished results from our laboratory also show that respiratory muscle from CH animals performs better in acute hypoxic conditions than muscles from normoxic control animals i.e. there is hypoxic tolerance. This is potentially beneficial to the muscle in
the short-term however structural and metabolic adaptations may result in poor clinical outcomes in the chronic diseased state (years), particularly with respiratory disease exacerbations and periods of dyspnoea.

1.6 Molecular Adaptations in Hypoxia

As outlined in section 1.5, there are several specific compartments and/or signalling cascades of the muscle fibre that may be affected in hypoxia. We must also be aware of secondary and tertiary stimuli resulting from hypoxia that may even compete/interact with hypoxia to influence phenotype changes in a hypoxic environment e.g. increased demands placed on the diaphragm. As mammals are generally less active in hypoxia [42], power-generating limb muscle (EDL) activity may be decreased compared to tonically active postural limb muscles (soleus) and may account for differences observed here.

1.6.1 Structural Adaptations

Fibre Type: Muscle fibre type proportions vary in accordance with activity. Slower fibres are more energy efficient, however they require a higher O$_2$ quantity to produce requisite ATP than fast fibres. Type II fibre transformation into the more enduring type I fibres has been observed in the severe COPD diaphragm [71,72] along with other slow sarcomere related proteins. There is contrasting literature regarding fibre type transitions in intercostal muscles [73,74]. Fibre type shifts in mild COPD are not as pronounced suggesting the adaptive process is time-dependent. Fatigue tolerance studies from our laboratory suggest that CH-induced respiratory muscle functional adaptations are time-dependent [22]. However, there is no significant change in fibre type proportions in diaphragm muscle after six weeks of CH [22,65,75]. Even from birth, when muscle plasticity is greatest, type I, IIa, IIb and IIx fibres remained unaltered in rat diaphragm after six weeks of CH; however, MHC isoforms changed significantly after thirty-nine weeks [76]. Hypoxia exposure in humans does not produce fibre type changes in vastus lateralis muscle [77]. Even high altitude natives do not present with differential fibre type distributions in vastus lateralis muscle compared to lowlanders [78]. Unfortunately, there are no studies to date on human respiratory muscle tissue fibre type composition after CH/high...
altitude exposure. Early-life exposure to CH can result in functional changes that persist into adulthood without fibre type adaptation [79]. Longer exposures may be necessary to induce detectable fibre type change. None the less, functional adaptations are observed in the respiratory muscles of animal models of CH prior to any potential fibre-type change. Moreover, the HIF transcription factors which are sensitive to hypoxia and influence fibre type transitions may have only minor roles in respiratory muscle functional adaptation to CH.

**Fibre cross-sectional area:** Fibre cross-sectional area (FCSA) modifications are consistently reported in the literature on hypoxia and the diaphragm muscle. McMorrow et al., report rat diaphragm fibre atrophy following six weeks of CH in type I, IIA, IIB, and IIX fibres (1410 ± 38 vs. 1232 ± 25; 1422 ± 26 vs. 1384 ± 23; 2565 ± 80 vs. 2040 ± 75; 5024 ± 177 vs. 3424 ± 85µm² mean ± SEM for control vs. CH respectively) [22] alongside no change in numerical density or areal density. Gamboa and Andrade report mouse diaphragm fibre atrophy following four weeks of CH (656 ± 59 vs. 488 ± 55 µm² mean ± SEM for control vs. CH respectively) [67]. Degens et al., report rat diaphragm fibre atrophy following four weeks of CH (data is presented in graphs rather than tables). This is especially interesting given that we might expect atrophy in non-working limb muscles compared to working respiratory muscles in hypoxia. Of course, decreases in FCSA will serve to reduce O₂ diffusion distances but may also decrease force generating capacity should myofibrils also decrease. Muscle fibre atrophy is observed in the diaphragm and limb muscles of severe COPD patients and animal models of CH [22,44,67,80,81] and in limb muscles of lowlanders who travel to high altitude [82], with the slow oxidative and fast oxidative type I and type II fibres affected most. In severe COPD patients, this phenotype in diaphragm muscle appears to be driven by reduced MyoD mRNA, increased MAFbx mRNA, and decreased Nedd4 mRNA (E3 ubiquitin ligases; MAFbx is up-regulated during muscle atrophy, Nedd4 is up-regulated during disuse but not catabolic states), and decreased myostatin mRNA but increased myostatin protein levels (inhibits muscle differentiation and growth) [81]. The atrophy signals are observed to be greater in limb muscles compared to the diaphragm in COPD patients [83] and not in controls indicating a role for local factors in hypoxia-induced atrophy. There is no difference between limb and diaphragm in hypertrophy signals.
in COPD or normal lung function patients with the exception of phosphorylated-(phospho)-protein 70 ribosome S6 kinase (p70S6K) content being greater in the COPD diaphragm. The limb muscles are clearly more at risk of atrophying than the diaphragm in COPD patients, however this may be due to decreased voluntary activity of the limb muscles in exercise intolerant patients. Unloading-induced atrophy is rapid, especially in weight-bearing muscles [84]. The proteasome complex degrades idle or damaged proteins. Increased proteasome activity has also been reported in the COPD diaphragm without a change in proteasome content indicating increased activity of individual proteasomes [85]. Furthermore, a proteasome inhibitor significantly improved contractile function and reduced myosin protein loss in emphysematous hamsters [86]. No significant changes to numerical fibre density or areal density have been reported for the diaphragm after six weeks of CH exposure [22,65]. Six weeks of CH induces hypertrophy of type IIa and IIx fibres in rat sternohyoid but no changes in EDL or soleus FCSA have been observed [22]. No change in mouse gastrocnemius and soleus FCSA was observed after four weeks of CH but a significant decrease was observed for diaphragm muscle [67]. The latter study [67] also presents a CH-induced left-shift in the frequency distribution of diaphragm fibres as functions of FCSA (i.e. larger to smaller CSA) while the frequency distribution for limb muscles became more concentrated at the centre (i.e. decreased number of smaller and larger CSA fibres and increased mid-range CSA fibres). Other studies have reported a significant decrease in the FCSA of type IIb fibres in rat EDL and soleus [65]. After three weeks of CH, there was no change in FCSA in rat EDL, soleus or tibialis anterior [75]. Neither was there any change to FCSA in mouse gastrocnemius, plantaris and soleus after four weeks of CH [67], however the FCSA of type IIa fibres was significantly decreased in rat soleus as was FCSA of type IIx fibres in rat EDL [66]. Four weeks of CH induced an overall decrease in FCSA, regardless of fibre type, in rat plantaris muscle [87]. It is clear that six weeks of CH induces fibre atrophy in the diaphragm muscle and plausible that the fibre type composition and workload influence the degree of fibre atrophy and the mechanism (mechanism is currently unknown).

Protein regulators of muscle fibre type composition, including MAPKs are mentioned in section 1.4. Muscle fibre size is also a defining feature of fibre type
and regulation of fibre size and type are intrinsically coupled by cross-talk between the MAPKs and the Akt signalling cascade. Progressive phosphorylation of the Akt protein and downstream targets such as mammalian target of rapamycin (mTOR) drives hypertrophy and forkhead box (FOXO)3a places a brake on atrophy [88–91]. The contribution of atrophic mRNA signals to COPD diaphragm atrophy suggests that the FOXO3a transcription factor and p38MAPK, which drive atrogin gene expression, will be activated in hypoxia. Hypoxia has also been shown to induce FOXO1 activity and expression, and a downstream activator of autophagy in the form of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) [80]. Furthermore, hypoxia increases Redd1 mRNA transcription, an inhibitor of the mTOR(C1) complex 1 pathway [92,93] suggesting that muscle atrophy is a combination of both active and inactive processes. However, others have shown that decreased protein synthesis does not appear to be a factor in hypoxia related muscle atrophy [94]. In fact protein synthesis rates are increased according to a study by Chaudhary et al. [94] although protein breakdown will provide substrate for protein synthesis. The ubiquitin-proteasome pathway’s activity accounts for the majority of muscle degradation in cancer cachexia where hypoxia-induced changes may play a role. Atrophy likely contributes to the functional adaptations discussed above and is a hypoxia-induced mechanism we explore further in the CH diaphragm (Chapter 5).

1.6.2 Metabolic Adaptations
Chronic increased sub-maximal activity of respiratory muscles in CH might be expected to increase their capacity for oxidative metabolism. Mitochondria, which are the major energy producers of the muscle cell, require oxygen to produce ATP and are likely to be affected in hypoxia. To complicate matters further, hypoxia and HIF-1α have been shown to promote a more glycolytic phenotype in cells [95]; and with the blood flow changes and body weight changes that occur, nutritional status and metabolic substrate availability could further confound metabolic regulation. Atrophy and protein degradation will also free amino acids which can substitute for more typical metabolic substrates when required. Ultimately, alterations to match energy production and expenditure are likely to occur to support the functional changes observed in animal models of CH.
**ATP Production** – Lowlanders spending time at high altitude/simulated high altitude present with reduced mitochondrial content and signs of muscle wasting and lipofuscin granule accumulation [80,94,96–99]. Curiously, superior aerobic performance is observed in human and animal populations native to high altitude despite paradoxical lower skeletal muscle oxidative capacity [100,101]. Cellular respiration is also lower in animal models of CH. State 3 respiration rates in CD1 mouse hind-limb skeletal muscle mitochondria were significantly lower compared to control after two days of CH exposure. This infers that ATP production is reduced after two days of hypoxia exposure. The altered respiratory control ratio values (RCR – ratio between state 3 and state 4 respiration) and impaired uncoupled respiration [102] indicates the electron transport chain itself must be damaged [103]. In contrast to acute hypoxia, mitochondrial respiration values after four weeks of CH are indicative of mitochondrial integrity in mouse diaphragm and hind-limb muscles, although state 3 respiration and oxygen consumption was significantly lower. There was no change in the RCR or uncoupled respiration [67]. Four weeks of CH decreased uncoupling protein (UCP)3 content in mouse diaphragm but not triceps surae [80]. Decreased UCP3 will decrease thermogenesis and facilitate optimisation of oxygen consumption by decreasing dissipation of the proton gradient. Mitochondria in diaphragm muscle are unquestionably distressed in times of CH albeit still functional.

Endurance training increases oxidative enzyme activity in skeletal muscle without major shifts in fibre type [18]. Consistent with the hypothesis of hypoxia-induced endurance training of the diaphragm – advanced COPD diaphragm fibres have higher succinate dehydrogenase (SDH) activity than controls but there is also a significant increase in type I fibres and decrease in type IIx fibres [104]. There is no change in oxidative/glycolytic ratio in the mild-to-moderate COPD diaphragm with the exception of the glycolytic enzyme lactate dehydrogenase (LDH), whose activity was decreased [105]. Severe, acute hypoxia decreases aconitase activity in mouse hind-limb [102]. Six weeks of CH has no effect on SDH or nicotinamide adenine dinucleotide phosphate reduced form (NADPH)-diaphorase enzyme activity in rat diaphragm or sternohyoid muscles [22]. Four weeks of CH exposure increases SDH activity in rat plantaris glycolytic fibres but not oxidative fibres [87], while a shift
from an oxidative to glycolytic phenotype was observed with high altitude exposure in the type 1 fibres from human vastus lateralis muscle [100]. Evidently, changes in metabolic enzyme activities are specific to the type, intensity and duration of the hypoxia stimulus and the muscle in which activity is measured. Regional adaptations in metabolism and ATP production could occur but remain hidden in global tissue measurements. Hypoxia in exercise decreases phosphocreatine recovery as well as decreasing pH levels in human gastrocnemius muscle [106,107]. Both of these stimuli will also cause fatigue in skeletal muscle [108]. Earlier recruitment of glycolytic fibres may be required inducing an increase in their oxidative capacity through activation. An additional 5% duration of recruitment has been shown to increase the oxidative capacity of a muscle cell [109]. Also, training during hypoxia increases the oxidative capacity of limb muscle [110–112]. While these studies use different metabolic enzymes as a measure of oxidative/glycolytic capacity, these enzymes may not be maximally active in vivo and a reserve capacity may be present should more metabolic substrate present itself. Furthermore, the catalytic activity of an enzyme under saturating conditions may not change while substrate flux according to availability and up-stream enzyme activity in metabolic pathways may be important.

In the diaphragm after CH, adaptations occur to improve ATP production despite damage to the mitochondria; however the full mechanism behind this is yet to be delineated. The effects of hypoxia on upstream metabolic pathways are particularly unclear and require elucidating. Overall, hypoxia decreases ATP production in respiratory muscle but given blood-flow changes, there isn’t likely to be a detrimental change in substrate availability. A thorough evaluation of key metabolic enzyme activities from a single study is required (Chapter 4).

**ATP Utilisation** – Hypoxia decreases ATP production in skeletal muscle, thus, tighter constraints on ATP usage will be important. Reflex hyperventilation makes the constraints on ATP usage of greater importance to the diaphragm muscle. Skeletal muscle ion pump ATPases are the largest consumers of ATP in the muscle cell and are thus candidate sites for regulation. The Na⁺/K⁺ ATPases establish the ionic concentration balance across the cell membrane, maintaining myocyte
excitability and regulating myoplasmic volume. Increased pump activity improves skeletal muscle endurance [113] whereas ouabain inhibition of the pump can accelerate muscle fatigability [114]. Six weeks of CH increased Na+/K+ ATPase pump content in rat diaphragm and EDL but not in sternohyoid or soleus muscles [22]. A decrease in pump content in human vastus lateralis was observed after a three weeks at high altitude [115] and eight weeks at high altitude which included intermittent exercise [112]. Changes in pH regulation including the requirement of the Na+/K+ ATPase to pump out excess Na\(^+\) that enters the cell via the Na\(^+\)/H\(^+\) exchanger (NHE) may also be important given that in a working muscle such as the diaphragm in hypoxia, local acidity is controlled for by NHE removing the produced H\(^+\). The sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) resides in the SR membrane. SERCA pumps transfer Ca\(^{2+}\) from the myoplasm to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation. SERCA1 is predominantly found in fast glycolytic fibre types and SERCA2a in slow oxidative fibres. Overload and chronic electrical stimulation increases SERCA2 and decreases SERCA1 protein expression in fast skeletal muscle [116,117]. Fatigue alterations in \(\frac{1}{2}\)-relaxation time also implicate the SERCA pumps in the fatigue process [118]. We might expect an increase in SERCA2 content in the CH diaphragm given the observed improved endurance [22]. One theory suggests fibre type transformation including SERCA isoform transformation: SERCA1/MHC IIa \(\rightarrow\) SERCA1+2/MHC IIa+MHC I \(\rightarrow\) SERCA2/MHC I. This fits with measurements of SERCA protein in severe COPD patient diaphragm [119]. Our laboratory has reported no significant difference in the areal density of fibres expressing SERCA2 in rat diaphragm after six weeks of CH exposure [22]. Myosin ATPase activity employs ATP hydrolysis to move myosin heads and pull actin filaments, thus shortening cross-bridges and generating force. This is regulated by the troponin complex. The troponin complex consists of three subunits: troponin C (TnC), troponin I (TnI), and troponin T (TnT). Ca\(^{2+}\) binding to the N-terminus of TnC triggers contraction by removing TnI inhibition of the mATPase. TnT is needed for Ca\(^{2+}\)-dependent regulation of the troponin complex as well as complete inhibition of mATPase activity. Significant decreases in TnC and TnI were observed in acute hypoxic mouse diaphragm preparations fatigued ex vivo compared to unfatigued control. The extraction of endogenous troponins from control fibres reduces maximal Ca\(^{2+}\)-activated force and
adding recombinant troponins to hypoxic-fatigued fibres improves maximal Ca\(^{2+}\)-activated force [68,120]. Hypoaxemia can induce post-translational modification of diaphragm TnI in anesthetized, spontaneously breathing canines [121]. Cross-bridge modifications may play a role in muscle adaptation to CH from both metabolic and functional perspectives.

Na\(^+\)/K\(^+\) ATPase and mATPase content are more likely candidates of adaptation with CH. The lack of fibre-type change, SERCA2a density change, and contractile kinetic changes [22] is in opposition to SERCA content changes or structural modifications. While Na\(^+\)/K\(^+\) ATPase content increases are in contrast to required metabolic modifications, the observed fibre atrophy is suggestive of mATPase decreases. However, as will be discussed in Section 1.7, structural modifications can occur to proteins that may limit their energy consumption.

### 1.6.3 Calcium Handling
Ca\(^{2+}\) is released through the RyR channels for contraction and re-uptake is through the SERCA proteins of the SR for muscle relaxation. There are a number of important intra-SR proteins that can signal information to the ion channels about the \([Ca^{2+}]\) in the SR and bind Ca\(^{2+}\) to reduce to intra-SR free [Ca\(^{2+}\)]. As well as being essential to contractile function, Ca\(^{2+}\) can initiate many myocellular signalling events through Ca\(^{2+}\) binding proteins such as CaM and calcineurin. Hypoxia significantly alters Ca\(^{2+}\) transients in C2C12 myotubes [122]. Ca\(^{2+}\) signalling events and Ca\(^{2+}\)-protein interactions may indirectly affect muscle function. Increased intracellular [Ca\(^{2+}\)] induces Na\(^+\)/K\(^+\) ATPase \(\alpha1\) mRNA expression via the calcineurin and CAM kinase II (CamKII) signalling cascades in rat skeletal muscle oxidative fibres [123]. This transcriptional response occurs through the nuclear factor of activated T cells (NFAT). NFAT also controls activity-dependent muscle fibre type specification [124], however there is no change observed to muscle-fibre type composition before functional changes emerge in CH.

### 1.6.4 The Sarcomere
The sarcomere is the cellular compartment where Ca\(^{2+}\) and ATP combine with a structure consisting of several regulatory proteins to produce muscle contraction. As
Ca\(^2+\) release and re-uptake are further down our list of candidates to be the direct mechanism behind respiratory muscle dysfunction in hypoxia, the cross-bridge becomes the logical cellular target, with altered sensitivity to Ca\(^2+\), less available ATP, and atrophy being the likely mechanisms. Degens et al., (2010) show a small change in Ca\(^2+\) sensitivity in the CH diaphragm so there may be sarcomere protein modification [70]. Muscle fibre atrophy, too, may affect the cross-bridge. Indeed, we have mentioned already the intricate links between hypoxia, Ca\(^2+\), sarcomere proteins, mATPase and muscle contraction.

1.6.5 Myokines

As mentioned in section 1.2, skeletal muscle is increasingly recognised for its role as a regulator of endocrine signalling. It has been suggested that secretion of myokines may facilitate the beneficial effects of exercise in chronic disease patients [125]. However, there is no literature to date on the role of myokines in hypoxia-induced diaphragm adaptations and thus are not a focus of this thesis.

1.7 Redox Homeostasis

Undoubtedly, there are a vast number of potential target sites for functional manipulation in skeletal muscle. Ultimately, adaptation will come down to modification of the protein component of the cell, whether through expression, structural alterations or manipulating a protein’s environment (pH, temperature etc.) to change sensitivity, activity or functional role to govern growth, metabolic activity and of course contractile performance. The field has considered fibre-type changes, fibre atrophy, metabolic changes including glycolytic/oxidative capacity and integrity of the respiratory chain in mitochondria, constraints on ATP usage, Ca\(^2+\) signalling, actin-myosin interactions in the sarcomere and pH. However, the mechanisms driving CH-induced molecular and functional remodelling including those upstream of processes already elucidated remains unclear. Moreover, the data is fragmented and in some parts contradictory, while some studies place too much physiological significance on stand-alone data e.g. a single metabolic enzyme’s activity as an index of metabolic capacity. If we simply ask the question of where the effect of hypoxia is likely to manifest in the first instance, the main candidates would
be the HIF transcription factors (O<sub>2</sub> results in degradation vs. hypoxic stabilisation) and mitochondria (O<sub>2</sub> is the ultimate electron acceptor in the respiratory chain). HIF-1α levels are unchanged following 7-9 days at high altitude in human vastus lateralis muscle [97] and potentially will not change in the long term given that the perceived hypoxia stimulus will decrease following acclimatisation. Fibre-type changes would also be driven by HIF, especially after chronic sustained HIF activation, yet these do not occur. Therefore, hypoxia effects are more likely to be observed at the mitochondrial level, which include increasing reactive oxygen species (ROS) formation [96,97,102,126], albeit somewhat paradoxically. A reduction in oxygen availability leads to a prevention of flow of electrons along the electron transport chain, subsequent stockpile of electrons and the ensuing release of O<sub>2</sub> from mitochondria [127–130] whereas anoxia prevents radical production as there is simply no oxygen to take part in the reaction. Furthermore, increased muscle activity increases ROS production [131–133] and the reflex hyperventilatory response to hypoxia increases diaphragm muscle activity.

### 1.7.1 Reactive Oxygen Species

Oxygen is one of the most electronegative elements. When partially reduced, as occurs in various chemical reactions, it forms ROS. Basic cellular components such as proteins, lipids and DNA are all at risk of damage from oxidative stress. ROS is linked to many debilitating diseases and are thought to be the main reason of aging. Some proteins are damage resistant and instead use oxidative modifications to change their activity and even protect the cells from oxidative stress. O<sub>2</sub>· and nitric oxide (NO) are the primary reactive species. Despite their high reactivity, ROS also undergo chemical change to alleviate reactivity via antioxidant defence mechanisms e.g. O<sub>2</sub>· has a higher reactivity with superoxide dismutase (SOD) than most molecules which catalyses the formation of H<sub>2</sub>O<sub>2</sub> (Fig.1.1). ROS and redox homeostasis changes, which have been associated with functional changes in CH and other models (and will be discussed in this section), represent a way in which function, metabolism and fibre atrophy processes may all be manipulated and are thus best candidates for driving CH-induced remodelling in the diaphragm muscle.
Figure 1.1: Superoxide and nitric oxide pathways of protein oxidation and antioxidant-induced reduction. $O_2^-$ may 1) be converted into hydrogen peroxide by the enzyme superoxide dismutase; 2) actively modify proteins by direct interaction; or 3) combine with nitric oxide to form the derivative peroxynitrite. Hydrogen peroxide may 1) chemically modify proteins directly; 2) be reduced to water and oxygen by the enzyme catalase or the cyclical antioxidant GSH system; or 3) take part in Fenton reactions to form the hydroxyl radical which is extremely reactive. GSH can also form disulphide bridges with oxidised protein thiols (glutathionylation – not shown in diagram); GSH = glutathione; GSSG = oxidised glutathione; GPx = glutathione peroxidase; GR = glutathione reductase;
Mitochondria are not the only source of ROS in the muscle cell (Fig. 1.2). Different models of skeletal muscle adaptation reveal a role for ROS generated from cytosolic oxidases such as xanthine oxidase and NADPH oxidase [90,134–137]. Several proteins using or producing NAD(P)H co-enzymes as reducing equivalents can produce ROS when substrate availability is altered or the proteins themselves become modified e.g. 2-oxoglutarate-dehydrogenase can produce ROS [138–141]. However, mitochondria in the hypoxic diaphragm are compromised [67,80] and as such are candidate to increase ROS production in the CH diaphragm.

Different paradigms of hypoxic stress have revealed an integral role for ROS in the respiratory control system. It has been reported that HIF and ROS play an important role in regulating the carotid body response to hypoxia [142–144]. Furthermore, there is a significant role for NADPH oxidase in chronic intermittent hypoxia induced modulation of the respiratory control centres in the brainstem and the phrenic nuclei [145]. It is entirely plausible that the main effector organs of respiratory control, the respiratory muscles, would be manipulated by alterations in redox balance in hypoxia. The pathogenesis and/or progression of several models of respiratory muscle dysfunction, such as sepsis, mechanical ventilation, muscular...
dystrophy etc., are also thought to be, at least in part, ROS mediated [16,146,147]. Atrophy, which is common to all of these models, is also closely associated with changes in ROS levels. For instance, FOXO3a and p38 MAPK transcription factors of atrophy genes are closely associated with ROS [148,149] and proteasome activity may be up-regulated with ROS [150]. Increased contractile activity will also stimulate ROS production [131–133]. We know that basal levels of ROS are required for function and even a slight shift toward a more oxidising environment is beneficial to performance [151] but too much ROS are detrimental to function [152] (see Fig. 1.3 below).

Redox modification of proteins affects their function. For example, actin oxidation is observed in several disease settings including COPD, Alzheimer’s disease and cardiac ischaemia [153–155]. Despite its complexity [156–160], however, actin-α oxidation is explicitly known to disrupt actomyosin interaction [161,162] and thus may affect muscle contraction directly.

1.7.2 ROS and Hypoxia
Further evidence to support the hypothesis of redox modifications in the diaphragm following CH exposure include mitochondrial morphological changes such as swelling and cristae degeneration which have been observed in rat [163] and human [102] skeletal muscle exposed to hypoxia. Four weeks of CH exposure increased activity of auto-phagocytic proteins (BNIP-3) and decreased PGC-1α and peroxisome proliferator-activated receptor gamma (PPARγ) protein resulting in decreased mitochondrial density and associated proteins in mouse diaphragm [80]. Both damage and resulting autophagy of mitochondria is potentially ROS mediated. Mitochondrial aconitase (an O$_2^-$ sensitive enzyme due to an iron-sulphur cluster) activity can be measured as an index for O$_2^-$ generation from mitochondria. Two days of hypoxia significantly decreased mitochondrial aconitase activity in CD-1 mice [102]. Additionally, Vitamin E, GSH and mitochondrial protein thiol content were decreased alongside increases in GSSG concentration, protein carbonylation and mitochondrial heat shock protein (HSP)-60 indicating the antioxidant defence system has been overwhelmed. Chronic nitric oxide synthase (NOS) blockade decreases Na$^+$/K$^+$ pump content and prevents CH-induced improved muscle
endurance in the rat diaphragm [22]. NO has both oxidant properties itself and antioxidant properties through the formation of peroxynitrite and as an \( \text{O}_2^- \) scavenger. Potentially it is through \( \text{Ca}^{2+} \) modulation that NO induces changes in \( \text{Na}^+/\text{K}^+ \text{ATPase} \) activity [123]. However, no change in protein nitrosylation has been observed in rat diaphragm muscle after six weeks of CH exposure [70]. In the COPD diaphragm and other respiratory muscles, oxidative stress and redox modification to key proteins whose function may be altered by redox modification has been observed [72,153,165,166] including those involved in metabolism, pH regulation and in contractile function directly.

![Graph showing relationship between isometric force and cellular redox state.](image)

**Figure 1.3**: Hypothesised and experimentally demonstrated relationship between isometric force of skeletal muscle and cellular redox state.

Image adapted and redrawn from [167].

*In vivo* and *ex vivo* pro/anti-oxidant drug treatment provides clues as to how exactly ROS influence muscle function. Tiron and tempol (exogenous antioxidants) prevent the increased rate of fatigue and reduction in force in Flexor Digitorum Brevis muscle measured *ex vivo* at 37°C compared to 22°C [168] through alterations in \( \text{Ca}^{2+} \) sensitivity. Temperature alone is not enough to alter \( \text{Ca}^{2+} \) sensitivity – a fatiguing stimulation is required. Also, application of dithiothreitol (DTT), a reducing agent, could reverse \( \text{Ca}^{2+} \) sensitivity changes [169]. Similar results have been observed in our laboratory in rat sternohyoid with tempol and tiron improving muscle performance *ex vivo* in hyperoxia and hypoxia [170]. Tiron improves rat diaphragm
endurance during acute hypoxia [171]. N-acetyl-cysteine (NAC) administration improves endurance in human respiratory and limb muscle and abolishes GSH oxidation [172,173]. NAC pre-treatment also attenuates the rise in plasma [K⁺] after exercise suggesting oxidative stress contributes to K⁺ deregulation. However, NAC treatment does not seem to inhibit high frequency fatigue in either reduced muscle preparations or human limb muscle [174]. Chronic NOS blockade decreases diaphragm Na⁺/K⁺ ATPase pump content and prevents CH-induced functional remodelling in the rat diaphragm [22]. Prolonged activity in skeletal muscle leads to an increase in nNOS expression [175]. After six weeks of CH exposure from birth, nNOS and eNOS protein expression increase in rat diaphragm [176] although after thirty-nine weeks of CH exposure from birth, they had decreased to approximately 20% and 15% of control levels respectively. Transgenic over-expression of catalase, CuZn-SOD or Mn-SOD causes morphological alterations in mouse diaphragm including an increase in non-contractile material and alterations to muscle fibre type. Specific force production is diminished yet fatigue tolerance during prolonged submaximal contractions is not improved [177]. However, improved fatigue tolerance has been reported in single muscle fibres of Mn-SOD overexpressing mice [178]. Although there is pharmacological evidence in favour of a role for NO in hypoxia-induced adaptation [22], no change in protein nitrosylation has been observed in CH muscle single fibres [70]. NO has also been shown to attenuate force production without altering Ca²⁺ sensitivity [179]. Of course, O₂⁻ interacts with NO to form peroxynitrite and so pharmacological modifications of NO availability would in turn affect the role of O₂⁻ in hypoxia. There is also conflicting evidence showing that NO reduces myofibril Ca²⁺ sensitivity in mouse skeletal muscle fibres via non-cGMP mediated pathways [180]. Conditions designed to block endogenous production of NO will also shift the voltage dependence of SR Ca²⁺ release activation towards more negative values [181]. In contrast, excess NO applied to isolated muscle preparation raises the [Ca²⁺], at rest with some SR Ca²⁺ release channels remaining locked open, which can be reversed with DTT [182]. NO is involved in altering the sensitivity of the SR Ca²⁺ release channel, in agreement with findings from other groups [183,184]. Decreases in SR Ca²⁺ release may be understood to be a safety mechanism. If SR Ca²⁺ release remains high when ATP levels are depleted due to increased activity, ATP might fall to a critical level where
functional mATPase, SERCA and Na+/K+ ATPase is lost resulting in rigor, breakdown of Ca\(^{2+}\) reuptake and loss of myocyte excitability respectively. Interestingly, PO\(_2\) appears to be a regulator of NO induced alterations in SR Ca\(^{2+}\) release channel activation with the redox state of several thiol residues acting as PO\(_2\) sensors [184]. Different concentrations of NO influence ryanodine effects on the SR Ca\(^{2+}\) release channel at different PO\(_2\) levels. Voltage dependence of RyR channel activation also occurs in the presence of H\(_2\)O\(_2\) [181]. H\(_2\)O\(_2\) induces inter-subunit cross-link disulphide bonds. Low levels of NO donors inhibit these cross-links and prevent activation of the RyR channel [185]. The two phenomena may be linked with NO providing protection against the effects of oxidation as is suggested by the study. Changes to Ca\(^{2+}\) sensitivity and decreased release of Ca\(^{2+}\) from the SR depend on ROS metabolism and SOD activity. In rats and Mn-SOD overexpressing mice, the decreased force observed after fatigue stimulation was due to decreased myofibrillar sensitivity as opposed to decreased Ca\(^{2+}\) release form the SR in wild type NMRI mice (~50% lower SOD activity) [178]. More intense oxidative insults were required to induce changes in calcium regulation and these changes could be abolished by adding a non-specific ROS scavenger [180,186]. ROS can also impede actin-myosin interactions [158,187].

It is clear that ROS can have widespread effects in regulating muscle function and tissue homeostasis. ROS are produced in hypoxia and with increased muscle contractile activity. They are also produced by – and contribute to – damaged mitochondria. The working diaphragm muscle in CH, unlike limb muscles, is potentially challenged by ROS on two fronts – by hypoxia per se and increased contractile activity. However, there have been no studies to date pursuing potential redox alterations to respiratory muscle proteins in an animal model of CH.
1.8 Hypothesis

We hypothesize that redox remodelling is pivotal in diaphragm muscle adaptation to CH.

The aim of this thesis is to elucidate molecular mechanisms driving diaphragm muscle adaptations to CH. A mouse model was utilised, exposed to different durations of CH in the presence or absence of chronic antioxidant supplementation. The sternohyoid, EDL, and soleus muscles were analysed alongside the diaphragm for comparative purposes. Protein redox changes and antioxidant changes are described in chapter 3. Redox-modified metabolic enzyme activity adaptations are described in chapter 4 alongside, metabolic enzyme activities at key substrate flux gating points, HIF-1α content, and succinate dehydrogenase activity at the muscle fibre level in a rat model of CH. Atrophy signalling and mechanism adaptations are described in chapter 5 and diaphragm muscle isotonic functional changes are described in chapter 6.
Chapter 2: Methods
2.1 Animal Model

All protocols involving animals described in this thesis were approved by local ethics committee and performed under licence from the Irish Government Department of Health and Children in accordance with EU legislation.

C57Bl6/J mouse models of CH were generated using environmental chambers (Oxycycler Model A84, BioSpherix Ltd, USA) where ambient oxygen levels can be tightly regulated (Fig. 2.1A). A reduction in the fractional volume of oxygen within the chamber results in a reduction in the fractional volume of oxygen in inspired air, airways, alveolus and ultimately blood with consequent hypoxemia for the animals. The Oxycycler permits user defined protocols to be used in several chambers simultaneously. A combination of oxygen and nitrogen gases are fed into the chambers and used to maintain the desired oxygen concentration. The chambers themselves contain oxygen sensors that continuously relay information back to the system software which facilitates immediate and appropriate gas infusion should oxygen concentration deviate from the user defined set-point. Animals are unrestrained and housed in their normal cages within the specialised chambers. Food and water were available ad libitum, and the chambers were maintained at room temperature with a 12/12 hour light/dark cycle. Once a week, the chambers were briefly opened to weigh animals and for cage cleaning. Soda lime and a molecular sieve (Sigma-Aldrich, Ireland) were placed inside the chambers to prevent CO₂ and condensation accumulation. An interesting note on the use of mouse models of CH: mice, like humans, but unlike rats (to the best of my knowledge), can be found at altitudes as high as 4,347m [188], and so mice and humans may be evolutionarily better equipped to deal with hypoxic insult.

2.1.1 Sample Size Calculation

Based on published work from McMorrow et al., (2011), a diaphragm fatigue index of ~72.5±6% from animals exposed to six weeks of CH was measured as significantly increased from the control animal fatigue index of ~50% ± 6% with n=6 animals per group [22]. The signal we want to be able to detect is ~22.5% and the standard deviation is ~14%. For experimental power to be 80% and sure of a
difference at the 5% significance level, the sample size required per group is \( \sim 7 \) (calculation below; 8 is used in case of animal loss during the experimental procedure).

\[
\text{Sample size} = \frac{(2*SD^2)*(Z_{a/2} + Z_{\beta})^2}{d^2}
\]

Where:
- \( SD = \) standard deviation;
- \( Z_{a/2} = Z_{0.05/2} = 1.96 \) taken from Z tables
- \( Z_{\beta} = Z_{0.2} = 0.842 \) taken from Z tables
- \( d = \) effect size

\[
(2*14^2)*(1.96+0.842)^2/(22.5^2) = 392*7.85/506.25 = 6.1
\]
\[
n = \sim 7
\]

2.1.2 Groups

Two independent CH exposure trials were carried out:

**Group 1**) Forty-eight (6 groups; n=8 per group) age- and weight-matched C576Bl/J mice (Charles River Laboratories, UK) were exposed to one, three, or six weeks of CH (FiO\(_2\)=0.1) or normoxia (3 separate groups for each of the time-points). The CH exposure trial for each group was completed as animals reached 12-13 weeks of age.

**Group 2**) The second cohort of thirty-two age- and weight-matched C576Bl/J mice (Harlan, UK) were exposed six weeks of CH (FiO\(_2\)=0.1) with/without chronic antioxidant supplementation (either tempol or NAC) in drinking water or normoxia (n=8 per group). Five fatalities were recorded during this second hypoxia trial: 2 x hypoxia, 2 x hypoxia + NAC, 1 x hypoxia + tempol. All fatalities occurred in different cages and different chambers and were the lightest mice of their respective cages. Furthermore, there were no signs of distress or fighting. We suspect a combination of the stress of hypoxia and being the runt of the group as the potential cause.
Figure 2.1: Oxycycler set-up and chamber oxygen profiles for treatment period.
Gas, Oxycycler, and Animal Chamber Set-Up are illustrated in (A); and (B) Oxygen profiles and durations of experimental groups.
2.1.3 Tempol and NAC
Tempol is a membrane-permeable antioxidant drug with properties similar to the enzyme SOD. It disproportionates the superoxide radical into hydrogen peroxide and water. Tempol has been observed to reverse the chronic intermittent hypoxia-induced decreased fatigue tolerance in rat diaphragm muscle [189] and reverse the chronic intermittent hypoxia-induced muscle weakness in the sternohyoid muscle [25]. It also increases force production in the sternohyoid muscle in acute hypoxia conditions [170,190]. In control conditions, tempol has no effect on/increases sternohyoid muscle force production [170,190]. NAC is a thiol-containing antioxidant drug that has intrinsic antioxidant properties itself and increases the cellular reserve of free radical scavengers by increasing GSH levels. It can be used to replenish GSH stores when they are depleted. It is sold as a nutritional supplement and used medically to treat paracetamol overdose. Furthermore, NAC is thought to possess some anti-inflammatory effects. NAC has been shown to reverse the chronic intermittent hypoxia-induced decreased fatigue tolerance and decreased peak specific force in rat diaphragm muscle [189]. NAC has no effect on sternohyoid muscle force in control conditions [170].

At the end of the gas treatment period, animals were anaesthetised by 5% isoflurane inhalation in air and euthanized by cervical dislocation. Diaphragm, sternohyoid, EDL and soleus muscles were excised, snap frozen in liquid nitrogen and stored at -80°C until further processing. Blood samples were taken in capillary tubes for haematocrit determination and the heart was removed for right and left (incl. septum) ventricular weight determination as indices of hypoxic (mal)adaptation. Results are presented in appendix 1.

2.2 Muscle Tissue Preparation

Frozen muscle samples were thawed on ice, weighed (for preparation only, muscle weights not recorded), and homogenised in ice-cold 10% w/v modified radio-immunoprecipitation assay (RIPA) buffer (1X RIPA, 200mM sodium fluoride, 1mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, phosphatase inhibitor cocktail (Fisher Scientific, Ireland) using a General Lab Homogeniser.
followed by centrifugation in a U-320R centrifuge (Boeckel & Co, Hamburg, Germany) at 13,000g for 15 min at 4°C to separate insoluble fractions from crude protein homogenate. Insoluble fractions (pellet) were discarded and crude protein homogenate (supernatant) was separated into aliquots and frozen at -80°C. Protein concentrations were evaluated using a bicinchoninic (BCA) protein assay (Pierce Biotechnology (Fisher Scientific), Ireland) against bovine serum albumin protein standards of known concentration. Samples are kept ice-cold throughout processing.

2.3 Redox Proteomics

2.3.1 Total Protein Carbonyl and Free Thiol Content
As previously described [191–193], muscle homogenates were incubated with either 2mM fluorescein-thiosemicarbazide (FTSC) or 2mM iodoacetamidofluorescein (IAF) (Sigma-Aldrich, Ireland) for two hours in the dark on ice for detection of free protein carbonyl and thiol groups respectively. Samples were then precipitated with 20% trichloroacetic acid (TCA) in acetone, followed by centrifugation at 11,000g for 3 min. Protein pellets were then washed with ice-cold excess 1:1 ethylacetate/ethanol or acetone (for FTSC and IAF respectively) to remove excess TCA, interfering salts and non-protein contaminants. Samples were dried, re-suspended in sample buffer (12.5% 0.5M Tris pH6.8, 25% glycerol, 2% sodium-dodecyl sulfate (SDS), a few grains of bromophenol blue) containing 5% beta-mercaptoethanol and heated at 95°C for 5min before electrophoretic separation on a 12% polyacrylamide gel. Fluorescent images of the gels were captured on a Typhoon Trio+ Variable-Mode Imager (GE Healthcare, UK). Protein bands were visualised by colloidal coomassie [194] and coomassie images were captured on a calibrating image densitometer (GS-800, Biorad, USA). This assay was performed on diaphragm, sternohyoid, EDL and soleus muscle samples from C57Bl6/J mice exposed to one, three, and six weeks of CH and normoxia controls.

2.3.2 2D Redox Proteomics
As previously described [191–193], this method separates proteins according to their isoelectric point and molecular mass such that they appear as spots when stained on...
polyacrylamide gels; protein spots can be analysed independently. Samples were treated as described above for 1D preparation until re-suspension in sample buffer. Samples were instead re-suspended in rehydration buffer (7M urea, 2M thiourea, 2% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 4% (v/v) ampholytes (Pharmalyte 3-10, Amersham, UK), 1% (v/v) destreak reagent (Amersham) and a trace amount of bromophenol blue) and loaded onto 70mm pH3-10 non-linear immobilised pH gradient (IPG) strips (GE Healthcare) in the dark and overnight. IPG strips were focused on a Protean isoelectric focusing (IEF) cell (Biorad) with linear voltage increases: 250V for 15min; 4000V for 2h; then up to 20,000Vh. Following IEF, strips were equilibrated (20 min) in equilibration buffer (6M urea, 0.375M Tris, pH 8.8, 2% (w/v) SDS, and 20% (v/v) glycerol) containing 2% (w/v) DTT, and then for 20 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. Equilibrated strips were then subjected to gel electrophoresis. Fluorescent and colloidal coomassie stained gel images were captured as described above. This assay was performed on diaphragm and sternohyoid muscle samples from C57Bl6/J mice exposed to six weeks of CH and normoxia controls.

2.3.3 Image Analysis
Quantity One image analysis software (Bio-Rad) was used to subtract background and quantify optical density for FTSC, IAF and colloidal coomassie labelling using the ‘rolling-disk’ method. For each sample, intensity of fluorescence was normalised to intensity of coomassie staining. For 2D separations, alignment of gels, spot matching, and quantification of spot volumes was carried out using Progenesis SameSpots image analysis software (Version 4.5; Non-linear Dynamics, Durham, NC, USA).

2.3.4 Protein Digestion and Identification
Gel spot selection criteria included statistical thresholding, separation, resolution, abundance and overlap in fluorescence-stain and muscle separations. Gel spots were used for in-gel protein digestion with trypsin. The extracted peptides were loaded onto a R2 micro column (RP-C18 equivalent) where they were desalted, concentrated and eluted directly onto a MALDI plate using α-cyano-4-hydroxycinnamic acid (5mg/ml) as matrix solution in 50% (v/v) acetonitrile and 5%
(v/v) formic acid. Mass spectra of the peptides were acquired with positive reflectron mass spectrometry (MS) and MS/MS modes using MALDI-TOF/TOF MS instrument (4800plus MALDI TOF/TOF analyser). The collected MS and MS/MS spectra were analysed in combined mode using Mascot (version 2.2; Matrix Science, Boston, MA) search engine and SwissProt (release 02_2013, 539 165 entries) database restricted to 50 ppm peptide mass tolerance for the parent ions, an error of 0.3 Da for the fragments, one missed cleavage in peptide masses, and carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. No taxonomy restrictions were applied. The identified proteins were only considered if a MASCOT score above 95% confidence was obtained (p < 0.05) and at least one peptide was identified with a score above 95% confidence (p < 0.05). This analysis was conducted by the Analytical Services Unit, Instituto de Tecnologia Química e Biológica (ITQB), New University of Lisbon, Lisbon, Portugal.

2.3.5 Statistical Analysis
Statistically significant differences in the total mean fluorescence intensity for 1D preparations and FTSC/IAF- and coomassie-labelled spot volumes (2D) were measured using Student’s t-test, Mann-Whitney test, or one-way ANOVA after testing for normality and equal variance in the data sets using Graph-Pad Prism (Graph-Pad Software Inc., CA, USA) and Progenesis SameSpots software for 1D and 2D preparations respectively. P<0.05 was the criterion for statistical significance.

2.4 Spectrophotometry

Light intensity at a specific wavelength, either changed by absorption (A) or produced by emission of light, can be measured by means of spectrophotometry. Different molecules absorb light with absorption peaks at specific wavelengths; thus, changes in the amount of molecules which absorb at a specific wavelength will be relative to the difference in light intensity (I_o-I_t) at that wavelength before and after passing through them. Increases in product or decreases in substrate related to an enzymes activity may be measured by this method, and when measured under
optimal conditions for that enzyme (e.g. pH, temperature, and saturating substrate) they may be used to compare a particular enzyme’s maximal catalytic activity in different tissues and in response to different stimuli, assuming the light-path length and volume of liquid is kept the same. Fig. 2.2A illustrates a simple spectrophotometer measuring absorption. The Beer-Lambert Law takes into account molar absorptivity and light-path length and can be used to calculate specific units of activity (Fig 2.2B). Molar absorptivity is a measurement of intrinsic absorption strength that depends on the nature of the absorbing species and the wavelength of the incident light. Further biochemical reactions can be used to form coloured products with strong absorbencies or to react with fluorescent or luminescent substrates that emit light which can amplify signals and provide greater experimental specificity. There are many enzymatic reaction substrates/products that peak in absorbance at a specific wavelength and are widely used e.g. common co-substrates for several metabolic enzyme reactions include dinucleotides such as NADH, which may be oxidised to form NAD$^+$ or produced by reduction of NAD$^+$. NADH readily absorbs light at 339nm whereas NAD$^+$ does not (Fig. 2.2C). Antioxidants, metabolic enzymes, proteasome and phospho-protein activities and content assays described below make use of these techniques. Unless otherwise stated, the Spectramax M3 (Molecular Devices, CA, USA) is the spectrophotometer used in the following experiments. Optimal protein concentrations were pre-determined by varying the protein concentration to find the appropriate linear range for enzyme activity measurements as a function of protein concentration.
Figure 2.2: Workflow and principles of spectrophotometry

A simplified diagram of a spectrophotometer measuring absorbance change is illustrated in (A); The Beer-Lambert Law is illustrated in (B); and peak absorbance wavelengths for the NAD$^+$ and NADH are illustrated in (C).
2.4.1 Antioxidants

2.4.1.1 Catalase Activity

The decomposition of H$_2$O$_2$ into oxygen and water in cells is catalysed by catalase, preventing both oxidative damage by H$_2$O$_2$ and the formation of the highly reactive hydroxyl radical. H$_2$O$_2$ peaks in absorbance at 240nm, and thus, the reaction in Fig. 2.3 can be followed by ultra-violet absorption spectrophotometry [195]. One unit of catalase activity decomposes one micromole of H$_2$O$_2$ per minute at 25°C and pH 7.0; 43.6 is the millimolar extinction coefficient of peroxide at 240nm. 30% H$_2$O$_2$ (Pharmacy Brand) was diluted in pH 7 phosphate buffer (0.75% v/v) and added to dH$_2$O, incubated for 5min in the spectrophotometer to achieve temperature equilibration. Sample was then added (33% H$_2$O$_2$ in final dH$_2$O and sample) and decreasing absorbance was recorded for 2-4min. The reaction velocity was taken from the initial linear part of the curve. Mouse liver sample and purified catalase (Sigma Aldrich) were used as positive controls and sodium azide (a catalase inhibitor) (Sigma Aldrich) was used as a negative control. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three, and six weeks of CH and normoxia controls.

\[
\text{Catalase} \quad 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

**Figure 2.3: Decomposition of hydrogen peroxide by the enzyme catalase**

2.4.1.2 Total Glutathione Content

GSH, the tri-peptide L-$\gamma$-glutamyl-L-cysteinylglycine, is an endogenous antioxidant and the most abundant non-protein thiol containing compound in cells. It is involved in amino acid transport, the formation of DNA precursors, the detoxification of xenobiotics, and protection from oxidative damage. It functions as a co-enzyme and can conjugate with many endogenous and foreign thiol radicals. It operates in a cyclical manner with the enzymes GPx and GR converting the oxidised GSH radical to the disulfide dimer form and reduction back to the singular compound form respectively. Total GSH content was measured using a colorimetric reaction in accordance with the kit manufacturer’s instructions (Caymen Chemical, USA).
sulfhydryl group of GSH in the sample is reacted with 5-5-dithio-bis-2-nitrobenzoic acid (DTNB or Ellman’s Reagent) to produce 5-thio-nitrobenzoic acid (TNB) and the mixed disulfide GSTNB. GSTNB is reduced by GR to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the recycling reaction which is directly proportional to the amount of GSH in the system (Fig. 2.4). The absorbance of TNB at 410nm provides an accurate estimation of GSH in the sample. To avoid interference from proteins containing sulfhydryl groups, samples are first deproteinated by addition of an equal volume of 10% metaphosphoric acid (Sigma Aldrich), incubation for 5min and centrifugation at 2,500g for 2 min with collection of the deproteinated supernatant at the end. 4M Triethanolamine (Sigma Aldrich) is added 1:20 to the sample to increase the pH before assaying. GSSG standards and samples are added to wells of a 96-well plate followed by addition of the assay cocktail (MES Buffer, Co-Factor mixture, Enzyme mixture and DTNB). Absorbance is measured after 25 min incubation in the dark on an orbital shaker. This assay was performed on diaphragm and sternohyoid muscle samples from C57Bl6/J mice exposed to six weeks of CH and normoxia controls.

Figure 2.4: Recycling mechanism of total glutathione assay
2.4.2 Metabolic Enzymes

2.4.2.1 Glucose-6-Phosphate Dehydrogenase Activity
Glucose-6-Phosphate Dehydrogenase (G6PD), which shuttles metabolic substrate between the glycolysis and pentose phosphate pathways to increase reducing equivalent availability for cells, is unique as it possesses dual co-substrate specificity. The G6PD assay is a modified version of that described by [196] and [197]. The reaction velocity is determined by measuring the increase in absorbance at $A_{339}\text{nm/min}$ resulting from the reduction of NAD or NADP. 55mM Tris·HCl buffer (pH 7.8) containing 3.3mM MgCl$_2$ was added to a 96-well plate (25% v/v) along with 60mM NAD (25% v/v) and 0.1M glucose-6-phosphate (25% v/v). The plate was incubated in a spectrophotometer at 30°C for 10min. Sample was added (25% v/v) and the increase in $A_{339}\text{nm}$ was recorded for 10min. $\Delta A_{339}/\text{min}$ was calculated from the initial linear portion of the curve. One unit reduces one micromole of NAD per minute at 30°C and pH 7.8; 6.22 is the millimolar extinction coefficient of NADH. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three and six weeks of CH and normoxia controls.

![Figure 2.5: G6PD catalyses the reversible oxidation of D-glucose-6-phosphate into 6-phospho-D-glucono-1,5-lactone](image)

2.4.2.2 Fructose-1, 6-bisphosphate Aldolase A Activity
Fructose-1, 6-bisphosphate Aldolase A (aldolase) is the isoform of the enzyme found predominantly in skeletal muscle, composed of four identical subunits, and is the 4$^{th}$ enzyme of the glycolysis pathway. It catalyzes the conversion of fructose-1-6-bisphosphate into both 3-phosphoglyceraldehyde and dihydroxyacetone phosphate (Fig. 2.5). This assay is based upon Boyer’s modification of the hydrazine assay [198] where 3-phosphoglyceraldehyde reacts with hydrazine to form a hydrazone.
which absorbs at $A_{240}\text{nm}$. 4mM fructose-1,6-bisphosphate (pH 7.5) (25% v/v), 0.03mM EDTA pH 7.5 (25% v/v), and 2.3mM hydrazine sulphate (25% v/v) were added to a 96-well plate and absorbance was recorded for 10min. Samples and a dH$_2$O blank (25% v/v) were then added to the plate and absorbance was recorded for another 10min. Using the linear portions of the curve, the $\Delta A_{240}/\text{min}$ of the blank was subtracted from the $\Delta A_{240}/\text{min}$ of the test. One unit is described as a change in absorbance of 1.00 per minute at 25°C and pH 7.5. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three and six weeks of CH and normoxia controls.

**Fructose-bisphosphate Aldolase-A**

Fructose-1,6-bisphosphate → Dihydroxyacetone phosphate + Glyceraldehyde-3-phosphate

NADH → NAD$^+$

![Figure 2.6: Aldolase catalyses the reversible reduction of fructose-1,6-bisphosphate into both 3-phosphoglyceraldehyde and dihydroxyacetone phosphate](image)

**2.4.2.3 Glycerol-3-Phosphate Dehydrogenase Activity**

Glycerol-3-Phosphate Dehydrogenase (G3PD) serves as a major link between carbohydrate and lipid metabolism as well as maintaining the inner mitochondrial membrane redox potential and being a major contributor of electrons to the respiratory chain. It catalyzes the reversible redox conversion of dihydroxyacetone phosphate to glycerol-3-phosphate (Fig. 2.6). 25mM Triethanolamine-HCL (pH7.5) containing 0.65mM EDTA and 0.025mM 2-mercaptoethanol (25% v/v) was added to a 96-well plate with sample (25% v/v) and dihydroxyacetone phosphate (25% v/v) and incubated for 10min in a spectrophotometer at 25°C. 0.03mM NADH (25% v/v) was then added to the mixture and $\Delta A_{339}$ was recorded for 20min. $\Delta A_{339}/\text{min}$ is taken from the linear portion of the curve. This assay was performed on diaphragm, muscle samples from C57Bl6/J mice exposed to one, three weeks and six of CH and normoxia controls.
2.4.2.4 Glyceraldehyde-3-phosphate Dehydrogenase Activity
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the 6th enzyme in the glycolysis pathway. Its activity catalyzes the conversion of glyceraldehyde-3-phosphate into D-glycerate-1,3-bisphosphate (Fig. 2.7). GAPDH activity was measured in a modified version of the assay described by [199,200]. Samples were added to 13.5mM sodium pyrophosphate buffer (pH8.5) containing 30mM sodium arsenate, 0.25mM NAD with 3.325mM DTT. Samples were incubated at 25°C for 10min to achieve temperature equilibration and to establish a blank rate, if any. 0.5mM DL-glyceraldehyde-3-phosphate was added and absorbance was recorded for 10min at A\textsubscript{339}nm. All chemicals above are described as final concentrations in the well. Measured rates were corrected by measuring the blank rate of the reaction. One unit is defined as 1µmol NADH generated/minute/mg sample. One unit is defined 1umol NADH generated/minute/mg sample. This assay was performed on diaphragm, sternohyoid, EDL and soleus muscle samples from C57Bl6/J mice exposed to one and three weeks of CH and normoxia controls.

Figure 2.7: G3PD catalyzes the reversible reduction of dihydroxyacetone phosphate into glycerol-3-phosphate

Figure 2.8: GAPDH catalyzes the reversible reduction of glyceraldehyde-3-phosphate into D-glycerate-1,3-bisphosphate
2.4.2.5 Lactate Dehydrogenase Activity
LDH catalyzes the reversible reaction of pyruvate to lactate oxidising the co-substrate NADH to the by-product NAD+ in the process (Fig. 2.9). Total activity is contributed to by five different isozymes which derive from two gene loci coding for two different polypeptides which combine together in groups of four to produce five different isozymes. LDH-4 and LDH-5 predominate in skeletal muscle. Total LDH activity was calculated as the decreasing absorbance at A_{339}nm produced by oxidation of NADH at 25°C and pH 7.3 in 0.2M Tris-HCL buffer containing 1mM sodium pyruvate and 0.22mM NADH. All chemicals above are described as final concentrations in the well. After 5min temperature equilibration and establishing a blank rate, if any, sample is then added and the reaction is recorded kinetically at 339nm for 10min. ΔA\textsubscript{339}/min was taken from the linear portion of the curve. This assay was performed on diaphragm, sternohyoid, EDL and soleus muscle samples from C57Bl6/J mice exposed to one and three weeks of CH and normoxia controls. Diaphragm muscle from six week groups was also assayed.

![LDH Catalysis Diagram](image)

Figure 2.9: LDH catalyzes the reversible reduction of pyruvate into lactate

2.4.2.6 Aconitase Activity
Aconitase activity was measured using a colorimetric reaction in accordance with the kit manufacturer’s instructions (Abcam, Cambridge, UK). Aconitase is a TCA cycle enzyme that catalyzes the isomerisation of citrate to isocitrate. Isocitrate, in this assay, undergoes further biochemical reaction resulting in a product that converts a nearly colourless probe into an intensely coloured form with a peak absorbance at A\textsubscript{450}nm. Samples are incubated in an activating solution containing cysteine-HCL and (NH\textsubscript{4})\textsubscript{2}Fe(SO\textsubscript{4})\textsubscript{2} on ice for one hour before addition to a 96-well plate in duplicate along with isocitrate standards. One half of the duplicate wells receive the sample reaction mixture (containing assay buffer, enzyme mix and substrate) and the other half the background mixture (containing assay buffer and enzyme mix only) and samples are incubated for 60min at 25°C. Developer is then added to each well.
and samples are incubated for a further 10 min. Absorbance is measured at A_{450} nm and background is subtracted from the test sample. One unit of Aconitase activity is the amount of enzyme that will isomerize 1 mmol of isocitrate per minute at pH 7.4 and 25°C. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three, and six weeks of CH and normoxia controls.

\[\text{Citrate} \xrightarrow{\text{Aconitase}} \text{Isocitrate} \]

\[\text{NADH} \xrightarrow{\text{NAD}^+}\]

**Figure 2.10: Aconitase catalyzes the reversible reduction of citrate into isocitrate**

### 2.4.2.7 Creatine Kinase Activity

In order to maintain an immediately accessible energy reservoir in skeletal muscle, creatine kinase (CK) catalyzes the phosphorylation of creatine. The highest catalytic concentration of CK is found in skeletal muscle. CK activity was measured using a colorimetric reaction in accordance with the kit manufacturer’s instructions (Abcam). In this assay, the generated phosphocreatine reacts with the CK enzyme mix to form an intermediate, which reduces a colourless probe to a coloured product with strong absorbance at A_{450} nm. Standards, samples and positive controls are added in duplicate to a 96-well plate. One half of each duplicate pair is incubated with a reaction mixture (containing assay buffer, enzyme mix, developer, ATP and substrate) and the other with a background mixture (containing assay buffer, enzyme mix, developer and ATP only) for 1 hour at 37°C and absorbance is recorded kinetically at A_{450} nm. Background is subtracted from test samples. One unit of CK is the amount of enzyme that will generate 1 mmol of NADH per minute at pH 9 and 37°C. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three, and six weeks of CH and normoxia controls.
Figure 2.11: Creatine Kinase catalyzes the reversible phosphorylation of creatine into phosphocreatine

2.4.3 Hypertrophy/Atrophy Signalling

2.4.3.1 Chymotrypsin-Like Proteasome Activity
Chymotrypsin-like activity of the 20S proteasome was measured by fluorometric spectrophotometry in accordance with the kit manufacturer’s instructions (Abcam). The reaction uses an AMC-tagged peptide substrate that releases highly fluorescent AMC in the presence of proteolytic activity. Jurkat cell lysate with high proteasome activity is supplied as the positive control and the proteasome specific inhibitor MG-132 permits differentiation of proteasome activity from other proteolytic activity which may be present in the sample. All samples and positive controls were assayed with/without the proteasome specific inhibitor on a white, 96-well plate. Samples, standards and positive controls are added to the plate. One half of each pair received the inhibitor and all wells except the standards received the substrate. Samples were incubated at 37°C and readings were taken kinetically for 1 hour at Ex/Em = 350/440nm. The first few minutes of the readings are discarded due to the time lag of the samples in reaching 37°C. Non-proteasome activity is subtracted from total activity and one unit of proteasome activity is defined as the amount of proteasome which generates 1 nmol of AMC per minute at 37°C. This assay was performed on diaphragm, sternohyoid, EDL and soleus muscle samples from C57Bl6/J mice exposed to six weeks of CH and normoxia controls. Diaphragm muscle samples from the one week exposure groups were also assayed.

2.4.3.2 Phospho-FOXO3a (Thr32) content
FOXO3a is a transcription factor which regulates multi-transcriptional targets involved in various cellular processes. This assay was performed in accordance with
the manufacturer’s instructions (Mesoscale Discovery, Gaithersburg, USA). Sample is loaded onto a 96-well plate containing specific capture antibodies bound to electrodes at the well bottom and the protein of interest is captured. The sample is then incubated in solution containing the detection antibody which is conjugated to an electrochemiluminescent label. Excess detection antibody is washed away and a read buffer is added to the wells which provide the optimum chemical environment for electrochemiluminescence emission and recording at 620nm. A specialised ‘Mesoscale’ spectrophotometer (MSD Sector Imager) was used to measure intensity of emitted light to provide a quantitative measure of phospho-FOXO3a in the sample. Inside the imager, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. As a mild and acute oxidative stress has been shown to induce activation of Akt signalling in neurons [201,202] and thus phosphorylation of FOXO3a, cultured primary Sprague-Dawley rat hippocampal neurons treated for 10min with H2O2 (courtesy of a neighbouring laboratory) were used as a positive control. Care was taken to use measurements in the dynamic range of the signal. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three, and six weeks of CH and normoxia controls.

2.4.3.3 mTOR and phospho-mTOR (Ser2448) content

The ‘Mesoscale Discovery’ technology facilitates the ability to quantify more than one protein in a single 96-well at one time. Several arrays of capture antibodies can be present in a well and light emitted based on temporal and spatial differences in the voltage applied to wells facilitate this multi-plex design. Using multi-plex assays, total mTOR and phospho-mTOR content were quantified in a similar method as phospho-FOXO3a. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three, and six weeks of CH and normoxia controls.

2.4.3.4 Phospho-Akt, phospho-p70S6K, phospho-GSK-3β, phospho-S6RP content

The phosphorylated content of Protein Kinase B (Akt), p70S6K, glycogen synthase kinase-3β (GSK-3β) and ribosomal protein S6 (S6RP) signalling proteins of Akt signalling cascade were measured using the Mesoscale multi-plex format. This assay
was performed on diaphragm muscle samples from C57Bl6/J mice exposed to one, three, and six weeks of CH and normoxia controls.

2.4.3.5 Phospho-ERK 1/2, phospho-p38, phospho-JNK content
The phosphorylated content of extracellular signal related kinase (ERK) 1/2, p38, and C-Jun-N-terminal kinase (JNK) MAPK was assessed using the Mesoscale multiplex format already described. Lysate from Jurkat cells treated with 1µmol rapamycin for three hours to activate MAPK phosphatase 1 was used as negative control and lysate from Jurkat cells treated with 50nM calyculin A and 200nM PMA for 15min to stimulate phosphorylation was used as a positive control. This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to six weeks of CH in the presence or absence of chronic supplementation with tempol or NAC and normoxia controls.

2.4.4 HIF-1α content
This assay was performed on diaphragm muscle samples from C57Bl6/J mice exposed to six weeks of CH ± chronic supplementation with tempol or NAC and normoxia controls using the Mesoscale multiplex format already described. Negative and positive controls are HeLa cells and HeLa cells treated with cobalt chloride (activates HIF) for 16 hours respectively.
Figure 2.12: Representation of method of protein content determination using the ‘Mesoscale’ assays

2.4.5 Statistical Analysis

Significant differences in enzyme activity and protein content assays were measured using Student’s t-test, Mann-Whitney test, one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc multiple comparison tests, or the Kruskal-Wallis test followed by Dunn’s post-hoc multiple comparison tests after testing for normality and equal variance in the data sets. P<0.05 was the criterion for statistical significance.

2.5 Isotonic Muscle Function

2.5.1 Ex Vivo Muscle Preparation

Animals were anaesthetised by 5% isoflurane inhalation in air and euthanized by cervical dislocation. The diaphragm and sternohyoid muscles were excised and placed in a bath of Krebs solution (NaCl 120mM, KCl 5mM, Ca^{2+} gluconate 2.5mM, MgSO_4 1.2mM, NaH_2PO_4 1.2mM, NaHCO_3 25mM, glucose 11.5mM, and 25mM d-tubocurarine) at room temperature and gassed with high oxygen (95% O_2/5% CO_2) before mounting in the test bath for performance assessment. Diaphragm muscle bundles (2-3mm at the rib end and slightly narrower at the central tendon end) were arranged with fibres running vertically between plate electrodes, anchored at the rib end to a fixed base and connected at the central tendon to a dual mode force transducer (Aurora Scientific, Canada) using thread. Whole mouse sternohyoid muscle bundles (1-2mm in diameter) were arranged between the electrodes with sternum anchored at the fixed base and at the hyoid bone to the force transducer so fibres are orientated vertically. Upon placement in the test bath, bundles were incubated at 35°C in Krebs solution gassed with high oxygen (95% O_2/5% CO_2). Muscle performance has previously been shown to be optimal under hyperoxic conditions (95% O_2) compared to normoxic conditions (21% O_2). Bundles were equilibrated for five minutes in gassed Krebs solution prior to initiating the experimental protocol. Sternohyoid was assessed first because unlike the diaphragm,
performance of the sternohyoid deteriorates when left for a prolonged period in the Krebs bath at room temperature (unpublished observation).

2.5.2 Protocol

*Peak isometric twitch and tetanic force and twitch contractile kinetics* Bundles were set to optimum length ($L_o$ – length at which peak twitch force occurs) by adjusting the position of the force transducer with a micro-positioner and stimulating with a single pulse until peak twitch force was reached. Twitch kinetics (time to peak (TTP) and half-relaxation time (T50)) were measured from the peak twitch force recording. After five minutes equilibration time, the force transducer was set to maximum rigidity (~500mN; >100% load) and a tetanic contraction was elicited by stimulating the bundle with supra-maximal voltage at 100Hz for 300ms. *Shortening length and velocity* The force transducer was set to minimum rigidity and contractions were elicited in incremental steps from 0-100% with one minute rest between each step. For example, when load was set to 20%, the muscle could only shorten when force production reached 20% of peak tetanic force. At the end of contraction, the muscle started to lengthen when force fell back below 20%. Shortening was determined as the maximum distance shortened over the whole contraction and shortening velocity was determined as distance shortened per the initial 30ms of shortening as this is when velocity is greatest. Peak shortening and peak shortening velocity were measured at 0% load. *Work and Power* Work and Power were determined at each step as the product of force x shortening and force x shortening velocity respectively. Peak work and peak power performance parameters were determined from the work-load and power-load relationship. *Fatigue* Five minutes after the step-test, muscle endurance was tested by repeated stimulation of the muscle at 100 Hz with 300ms trains every 2 seconds for a period of 5 minutes in isotonic conditions at 33% load. 33% load has previously been shown to elicit maximum power.

2.5.3 Data Analysis

Peak specific force ($F_{\text{max}}$) was calculated in N/cm$^2$ of muscle CSA. CSA was calculated as the blotted dry muscle bundle weight divided by the product of $L_o$ and the specific density, assumed to be 1.056g/cm$^3$. Peak specific shortening ($S_{\text{max}}$) was calculated as length shortened per optimal length ($L/L_o$). Peak specific shortening
velocity ($V_{\text{max}}$) was calculated as \( L / s \). Peak specific mechanical work ($W_{\text{max}}$) was calculated as Joules/cm\(^2\) and peak specific power ($P_{\text{max}}$) as Watts/cm\(^2\). For the assessment of isotonic fatigue, shortening, shortening velocity, work, and power were measured every 10s for the initial 2min and every 30s thereafter. After testing for normality and equal variance in the data sets, statistical comparisons were performed between groups using one-way ANOVA, Kruskal-Wallis test, two-way ANOVA, Tukey’s post-hoc multiple comparison tests, Bonferroni’s post-hoc multiple comparison tests, Dunn’s multiple comparison tests as appropriate using Graph-Pad Prism. P<0.05 was the criterion for statistical significance.

2.6 Succinate Dehydrogenase Histochemistry Analysis

SDH is a TCA cycle enzyme and also part of complex II of the respiratory chain. SDH catalyses the conversion of succinate into fumurate, with reduced flavin adenine dinucleotide (FADH\(_2\)) produced as a by-product of this reaction. FADH\(_2\) and, thus electrons are thereby provided to the respiratory chain for oxidative phosphorylation. In a previous study by our laboratory, diaphragm and sternohyoid muscle SDH activity in a rat model of CH was determined for the whole muscle composite section on slides by histochemistry and optical density measurement [22]. No significant differences were observed between control and CH in diaphragm or sternohyoid muscles. We sought to re-examine these slides as changes may have occurred at the fibre level that may be hidden in whole muscle analysis. Male Wistar rats were exposed to hypobaric CH (380mmHg) for six weeks [22]. Post treatment, diaphragm and sternohyoid muscles were excised and snap frozen. Transverse sections from each muscle were cryo-sectioned and SDH histochemistry was performed [22]. Scion Image was the software used for determination of SDH activity optical density per fibre cross sectional area.

K-means cluster analysis was performed on area-density plots of muscles from control and CH animals. K-means clustering partitions ‘x’ number of observations into ‘k’ number of clusters by iterative fine-tuning until all observations are grouped into the cluster of the nearest mean. Three means/clusters (k=3) were decided appropriate (small, middle, and large FCSAs). Three means are selected at random
and data points are grouped accordingly. The centroid of each cluster becomes the new mean and data points are grouped according to these new means. This process is repeated until the cluster centroids no longer change with the three most spatially distinct clusters remaining. SDH activity of fibre clusters in normoxic and CH sternohyoid and diaphragm muscles were compared.
Chapter 3: Redox remodelling in diaphragm muscle following chronic sustained hypoxia
3.1 Chapter Introduction

Mitochondria in the hypoxic diaphragm are compromised [67,80] and as such may be producing increased ROS – progressive to mitochondrial damage. Hypoxia increases ROS formation in several tissues [102,126] through increased electron leak from mitochondria [129,130]. Increased contractile activity will also stimulate ROS production [131–133]. Perturbations to ROS and redox homeostasis, which are associated with muscle functional changes in CH and other models, represent a process whereby function, metabolism and fibre atrophy in the diaphragm may all be manipulated (discussed in chapter 1). Oxidative stress correlation with human disease is widely accepted. COPD patients present with redox remodelling in their diaphragm muscle [72,153]. Increased protein oxidation has also been detected in respiratory muscles in response to chronic resistive loading, after several hours of mechanical ventilation, and in response to sepsis [16,19,203,204]. However, there is a paucity of information concerning how and where ROS mediate their effects.

Proteins represent the major non-water constituent of the cell and thus absorb the majority of ROS produced by the cell. This in turn will alter protein expression, through gene induction or targeted degradation, and/or affect catalytic function by altering a given protein’s covalent structure, both of which could impact homeostasis in the muscle proteome in times of oxidative stress. Protein oxidation may occur in a number of ways, with carbonylation being the most common form and free thiol oxidation being quite specific [159,205]. Many structural, metabolic, and stress response proteins can have their expression and functions modified by oxidation [72,102,184,204,206–211]. They may not all strictly be maladaptive as advantageous adaptations have been observed in models of oxidative stress e.g. metabolic enzymes in yeast that experience oxidant stress [212].

Carbonyls are functional groups of amino acids that contain a carbon atom double-bonded to an oxygen atom (C=O) e.g. ketones and aldehydes. Carbonylation is for the most part irreversible and carbonylated proteins are targeted for degradation by the 20S proteasome [213–218]. It has even been suggested that proteolysis might be a better indicator of oxidative stress than lipid peroxidation [216]. Further
carbonylation can result in aggregate formations that cannot be degraded, as occurs with aging [219,220]. Despite carbonylation being the most common form of protein oxidation [159,221], specificity can occur in many cases and consequently be conducive to protein quality control. Carbonylation may be physiologically important in targeting irreparably damaged protein for degradation rather than chaperoning and re-folding [159]. Enzymes that are idle due to a lack of substrate also appear more prone to carbonylation e.g. there is specific carbonylation of glutamine synthetase during nitrogen starvation [213,214,222]. A thiol is a carbon bonded sulfhydryl group (-SH). Free thiols are sulfhydryl groups that are exposed and oxidant sensitive. They are often found in enzyme catalytic sites and contribute to catalytic activity. Oxidation of protein thiol groups to reversible and irreversible protein disulphide bridges (R-SS-R), glutathionylated thiols (R-SSG), nitrosothiols (-SNO), and sulfoxides (thiol radical (-S)→ sulfenic acid (-SOH) → sulfonic acid (-SO₂H) → sulfonic acid (-SO₃H); with progressive oxidation) is highly specific in accordance with the low abundance of the amino acid cysteine in comparison to other amino acids [205].

In this chapter we describe protein carbonyl and free thiol oxidation in respiratory and limb muscles after one, three and six weeks of hypoxia compared to controls. We use redox proteomics to identify proteins that are redox modified in the diaphragm and sternohyoid after six weeks CH (a collaborative study with Prof Dave Sheehan’s Proteomic Research Group in the School of Biochemistry & Cell Biology, University College Cork). The approach also facilitates the assessment of protein expression changes. We hypothesize that differential and temporal changes in protein oxidation occur in respiratory and limb muscles. Furthermore, proteins key to muscle function will be redox modified in the respiratory muscles.

Protein redox changes may result from either increased production of ROS, decline in the antioxidant defence system, diminished capacity for removal of oxidized proteins, increased susceptibility of proteins to oxidative attack or increased proteins available for carbonylation such as those containing metals like Ferritin. Of course, these possibilities are not mutually exclusive [205]. A diminished capacity for protein removal is unlikely given that fibre atrophy is observed in this model, while
Ferritin will be required for increased haematocrit levels in hypoxia. Bearing this in mind, we are also interested in what happens to the antioxidant defence system of the cell.

Cells have evolved a host of antioxidant defence mechanisms including utilising specific enzyme activities (e.g. catalase) and non-protein reducing agents (e.g. GSH, Vitamin E) as well as having antioxidant responsive genes. Antioxidants inhibit reactive intermediates by being oxidised themselves.

We measured total GSH content in the respiratory muscles after six weeks of CH and catalase activity in the diaphragm after one, three and six weeks of CH. GSH is the most abundant non-protein thiol in mammalian cells and is present in concentrations of 0.5–10mM. It plays a role in many biological processes, including synthesis of proteins and DNA, transport of amino acids, and as an antioxidant. It may also form disulphide bridges with other oxidised thiols of proteins and lipids, in some cases conferring protection against further oxidative stress. It may also be used as a reductant in keeping other endogenous antioxidants such as Vitamin E in their reduced form. Catalase reduces H$_2$O$_2$ to water and oxygen and has an extremely high turnover rate. Catalase can be differentially expressed or activated in times of oxidative stress. We hypothesized that hypoxia increases antioxidant defences to combat oxidative stress.

### 3.2 Chapter Results

#### 3.2.1 Section A: Total Protein Carbonyl and Free Thiol Content

Fig. 3.1 illustrates the differences in the redox proteome between diaphragm and sternohyoid muscles in control animals before quantitative measurement. It is entirely plausible that exposure to hypoxia will result in differences in protein redox modification in respiratory and in limb muscles.
Figure 3.1: Representative images of 1D gel protein separations

The gel images are of labelled protein free thiols in control diaphragm samples (A) and control sternohyoid samples (B). Molecular weights cannot be displayed for the protein free thiol gel image - only the coomassie stained gel image. Despite this, the same range of protein weights which are displayed on each gel facilitates noting differences in protein free thiol content densities at different weights between both muscles.
3.2.1.1 Diaphragm
Temporal changes in diaphragm protein carbonyl and free thiol content are shown in Fig. 3.2. There are significant increases in diaphragm protein carbonylation after three and six weeks of CH (121.66 ± 16.91 vs. 91.32 ± 13.09, 81.09 ± 25.19 vs. 229.36 ± 35.46*, 70.72 ± 11.71 vs. 526.752 ± 72.42*** mean ± SEM normalised fluorescence intensity; control vs. CH; diaphragm protein carbonyl content after one, three, and six weeks of CH respectively; n=5-8 per group; *p<0.05, ***p<0.001) – indicative of progressive protein oxidation. Changes in diaphragm protein free thiol content, in contrast to carbonyl content, are bi-phasic. There are significant increases after one and three weeks of CH however this is significantly lower than control after six weeks (30.77 ± 5.33 vs. 281.65 ± 32.18***, 11.36 ± 1.34 vs. 142.61 ± 17.18***, 16.71 ± 1.8 vs. 9.97 ± 0.86** mean ± SEM normalised fluorescence intensity; control vs. CH; diaphragm protein free thiol content after one, three, and six weeks of CH respectively; n=7-8 per group; **p<0.01, ***p<0.001) – further indication of progressive oxidation although the findings observed after one week are suggestive of an early antioxidant response.

3.2.1.2 Sternohyoid
Sternohyoid protein carbonyl content and free thiol content changes after one, three, and six weeks of CH are shown in Fig. 3.3. Similar to diaphragm muscle, there are significant progressive increases in sternothyroid protein carbonyl content after three and six weeks of CH (313.88 ± 54.78 vs. 197.40 ± 46.90, 341.01 ± 96.95 vs. 1035.41 ± 38.92***, 222.94 ± 28.37 vs. 930.04 ± 75.72*** mean ± SEM normalised fluorescence intensity; control vs. CH; sternothyroid protein carbonyl content after one, three, and six weeks of CH respectively; n=5-8 per group; ***p<0.001). Changes in sternothyroid protein free thiol content are also bi-phasic. There are significant increases after one and three weeks of CH however this is significantly lower than control after six weeks (82.07 ± 9.87 vs. 203.91 ± 30.53*, 68.72 ± 4.91 vs. 128.69 ± 15.06*, 71.79 ± 2.02 vs. 13.92 ± 1.63** mean ± SEM normalised fluorescence intensity; control vs. CH; sternothyroid protein free thiol content after one, three, and six weeks of CH respectively; n=7-8 per group; *p<0.05, **p<0.01).
Figure 3.2: Diaphragm protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls. (A) Diaphragm protein carbonyl content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised fluorescence intensity; n=5-8 per group; (B) Diaphragm protein free thiol content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised fluorescence intensity; n=7-8 per group; *p<0.05, **p<0.01, ***p<0.001, ns=not significant; Student’s t-test or Mann-Whitney test as appropriate; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Figure 3.3: Sternohyoid protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls. (A) Sternohyoid protein carbonyl content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised fluorescence intensity; n=5-8 per group; (B) Sternohyoid protein free thiol content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised fluorescence intensity; n=7-8 per group; *p<0.05, **p<0.01, ***p<0.001, ns=not significant; Student’s t-test or Mann-Whitney test as appropriate. Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
3.2.1.3 EDL
Unlike the respiratory muscles, there are no significant changes to protein carbonyl content in EDL muscle after CH exposure (Fig. 3.4A) (113.04 ± 11.32 vs. 92.22 ± 14.94, 137.05 ± 49.04 vs. 219.19 ± 43.39, 86.35 ± 8.01 vs. 104.86 ± 20.75 mean ± SEM normalised fluorescence intensity; control vs. CH; EDL protein carbonyl content after one, three, and six weeks of CH respectively; n=4-8 per group). There are, however, significant increases in EDL free thiol content after one, three, and six weeks of CH (79.39 ± 24.18 vs. 491.25 ± 101.6**, 74.69 ± 12.59 vs. 232.87 ± 32.87***, 55.69 ± 3.39 vs. 185.33 ± 17.64*** mean ± SEM normalised fluorescence intensity; control vs. CH; EDL protein free thiol content after one, three, and six weeks of CH respectively; n=6-8 per group; **p<0.01, ***p<0.001) – the magnitude of the increase is seen to decrease over time (Fig. 3.4B).

3.2.1.4 Soleus
Similar to the respiratory muscles, but unlike the EDL, there is no change in protein carbonyl content in the soleus muscle after one week of CH but significant increases are observed after three and six weeks of CH (Fig. 3.5A) (227.73 ± 87.88 vs. 188.54 ± 46.96, 376.21 ± 28.46 vs. 613.59 ± 103.50*, 251.52 ± 76.81 vs. 980.61 ± 145.10** mean ± SEM normalised fluorescence intensity; control vs. CH; soleus protein carbonyl content after one, three, and six weeks of CH respectively; n=4-8 per group; *p<0.05, **p<0.01). Soleus free thiol content is significantly increased after one, three and six weeks of CH compared to controls (Fig. 3.5B) (175.33 ± 39.11 vs. 1874.80 ± 271.14**, 198.29 ± 24.35 vs. 615.65 ± 110.05***, 115.06 ± 9.51 vs. 279.62 ± 20.16*** mean ± SEM normalised fluorescence intensity; control vs. CH; soleus protein free thiol content after one, three, and six weeks of CH respectively; n=8 per group; **p<0.01, ***p<0.001); consistent with EDL there is a decline in increased free thiol content in the soleus with progressive CH.
Figure 3.4: EDL protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls. (A) EDL protein carbonyl content (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as normalised fluorescence intensity; n=4-8 per group; (B) EDL protein free thiol content (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as normalised fluorescence intensity; n=6-8 per group; *p<0.05, **p<0.01, ***p<0.001, ns=not significant; Student’s t test or Mann-Whitney test as appropriate; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Figure 3.5: Soleus protein carbonyl and free thiol content after one, three and six weeks of sustained hypoxia compared to normoxic controls. (A) Soleus protein carbonyl content (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as normalised fluorescence intensity; n=4-8 per group; (B) Soleus protein free thiol content (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as normalised fluorescence intensity; n=8 per group; *p<0.05, **p<0.01, ***p<0.001, ns=not significant; Student’s t test or Mann-Whitney test as appropriate; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
3.2.1.5 Total Protein Carbonyl and Free Thiol Content expressed as percentage of control

As illustrated in Fig. 3.6, one week of CH has no effect on protein carbonyl content in any of the respiratory or limb muscles studied, while three and six weeks induces progressive increases in both respiratory muscles and the soleus muscle. Taking the respiratory muscles as a separate group, there is greater carbonylation observed in diaphragm which has the greater complement of slow oxidative fibres (~7.5 fold and ~4.2 fold change in diaphragm and sternohyoid after six weeks of CH respectively) and greatest carbonylation is observed in the slow oxidative soleus muscle within the limb group (~3.9 fold vs. ~1.2 fold change in soleus and EDL after six weeks of CH respectively). Considering respiratory versus limb muscles, there is greater carbonylation observed in the working respiratory muscles than in the non-working limb muscles. A combination of muscle fibre type composition and workload are evidently involved in determining protein carbonylation in muscle after CH.

As illustrated in Fig. 3.7, one week of CH significantly increases protein free thiol content in each of the respiratory and limb muscles studied suggesting this may be a direct consequence of hypoxia *per se*. The greater fold change (~10.7, ~9.2, ~6.2, and ~2.5) is observed in soleus > diaphragm, > EDL > sternohyoid – again indicative of slow vs. fast fibre differences and respiratory vs. limb muscle differences. After three weeks of CH, protein free thiol content is significantly increased in all muscles although the fold change has decreased in all muscles except the diaphragm. After six weeks of CH, a respiratory vs. limb muscle difference is again observed with the respiratory group presenting with evidence of protein oxidation compared to controls.
Figure 3.6: Skeletal muscle protein carbonyl content expressed as a percentage of control after one, three, and six weeks of chronic sustained hypoxia. Diaphragm, sternohyoid, EDL and soleus protein free thiol content (mean ± SEM) expressed as percentage of control after one (A), three (B), and six (C) weeks of CH; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Figure 3.7: Skeletal muscle protein free thiol content expressed as a percentage of control after one, three, and six weeks of chronic sustained hypoxia.

Diaphragm, sternohyoid, EDL and soleus protein free thiol content (mean ± SEM) expressed as percentage of control after one (A), three (B), and six (C) weeks of CH; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1).
3.2.2 Section B: Redox Proteomics

In the diaphragm, 498, 264 and 996 resolved spots were matched in FTSC-, IAF-labelled and coomassie-stained separations respectively. A significant relative volume difference is observed in 34 FTSC, 23 IAF and 32 coomassie spots comparing control and CH groups (p<0.05). In the sternohyoid, 498, 264 and 996 resolved spots were matched in FTSC-, IAF-labelled and coomassie-stained separations respectively. A significant relative volume difference is observed in 87 FTSC, 51 IAF and 156 coomassie spots comparing control and CH groups (p<0.05). Proteins were selected for MS analysis based on separation, resolution, abundance, and overlap in fluorescence-stain and muscle separations. Protein smears and gel defects were excluded. Results for diaphragm and sternohyoid protein remodelling are presented in Table 3.1 and Table 3.2 respectively with proteins grouped according to cellular location and/or function. Selected spots can be visualised on the representative coomassie stained gels shown in Fig. 3.8. A change in FTSC and IAF fluorescence intensity signal independent of, or differential to, coomassie signal is indicative of protein redox remodelling. 88 spots were picked in total; 9 FTSC, 15 IAF, 16 coomassie - diaphragm; 12 FTSC, 12 IAF, 24 coomassie – sternohyoid; 63 spots were sent for identification with 59 successful identifications. Proteins identified include metabolic enzymes; stress response proteins that are associated with the mitochondria, the cytoplasm and the cross-bridge apparatus; as well as cross-bridge proteins and Ca\(^{2+}\) handling proteins. It should also be noted that just because a specific protein is not accounted for in the tables below, it does not mean that it is not being redox modified in CH.
Figure 3.8: Representative image of 2D-PAGE protein profiles after Coomassie staining of proteins. Diaphragm protein profile is presented in (A) and sternohyoid in (B). A molecular mass marker ranging from 14-116kDa is shown for size reference and isoelectric point is indicated along the range pH 10-3. Spot numbers match those presented in table 3.1 and table 3.2.
Table 3.1: Identification of proteins undergoing significant redox remodelling in the diaphragm muscle after six weeks of sustained hypoxia.

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein</th>
<th>Mw (Da)</th>
<th>GI number</th>
<th>Mascot score</th>
<th>MS/MS Matched peptides</th>
<th>Sequence Coverage</th>
<th>Carbonyl p-value, fold</th>
<th>Free thiol p-value, fold</th>
<th>Expression p-value, fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytochrome bc-1 complex subunit 1</td>
<td>52,819</td>
<td>341941780</td>
<td>430</td>
<td>6</td>
<td>26%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01, +1.2</td>
</tr>
<tr>
<td>2</td>
<td>ATP Synthase subunit α</td>
<td>59,716</td>
<td>416677</td>
<td>1370</td>
<td>12</td>
<td>48%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.05, +1.2</td>
</tr>
<tr>
<td>3</td>
<td>ATP Synthase subunit β</td>
<td>56,266</td>
<td>20455479</td>
<td>736</td>
<td>6</td>
<td>49%</td>
<td>&lt;0.01, +1.3</td>
<td>-</td>
<td>&lt;0.01, +1.3</td>
</tr>
<tr>
<td>4</td>
<td>Electron Transfer Flavoprotein subunit</td>
<td>27,606</td>
<td>92090596</td>
<td>648</td>
<td>6</td>
<td>47%</td>
<td>-</td>
<td>&lt;0.01, +1.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Aconitase hydratase</td>
<td>85,410</td>
<td>60391212</td>
<td>1260</td>
<td>9</td>
<td>47%</td>
<td>-</td>
<td>&lt;0.05, +1.3</td>
<td>&lt;0.01, -1.7</td>
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<tr>
<td>6</td>
<td>2-oxo-glutarate dehydrogenase, mt</td>
<td>116,375</td>
<td>14634547</td>
<td>477</td>
<td>4</td>
<td>22%</td>
<td>-</td>
<td>&lt;0.05, -2</td>
<td>&lt;0.01, -2.7</td>
</tr>
<tr>
<td>Glycolysis</td>
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<td>Fructose bis-phosphate aldolase A</td>
<td>39,331</td>
<td>113607</td>
<td>446</td>
<td>3</td>
<td>49%</td>
<td>-</td>
<td>&lt;0.05, +1.3</td>
</tr>
<tr>
<td>8</td>
<td>Triose phosphate isomerase</td>
<td>26,673</td>
<td>75070019</td>
<td>966</td>
<td>9</td>
<td>62%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01, -1.5</td>
</tr>
<tr>
<td>9</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>35,787</td>
<td>120702</td>
<td>679</td>
<td>5</td>
<td>39%</td>
<td>&lt;0.01, +1.4</td>
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<td>-</td>
</tr>
<tr>
<td>10</td>
<td>β enolase</td>
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<td>12206517</td>
<td>843</td>
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<td>51%</td>
<td>&lt;0.01, -1.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Pyruvate kinase isozymes M1/M2</td>
<td>57,303</td>
<td>146345448</td>
<td>6</td>
<td>53%</td>
<td>&lt;0.01, -1.2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Phosphagen and Lipid metabolism</td>
<td>12</td>
<td>Glycerol-3-phosphate dehydrogenase [NAD(+)]</td>
<td>37,548</td>
<td>121557</td>
<td>679</td>
<td>5</td>
<td>39%</td>
<td>&lt;0.01, +1.5</td>
<td>-</td>
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<tr>
<td>13</td>
<td>Fatty acid binding protein</td>
<td>14,810</td>
<td>20141333</td>
<td>767</td>
<td>5</td>
<td>60%</td>
<td>-</td>
<td>&lt;0.05, +1.1</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Creatine kinase s-type</td>
<td>47,355</td>
<td>125313</td>
<td>431</td>
<td>5</td>
<td>38%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01, -1.3</td>
</tr>
<tr>
<td>15</td>
<td>Creatine kinase m-type</td>
<td>43,014</td>
<td>124056470</td>
<td>931</td>
<td>8</td>
<td>54%</td>
<td>&lt;0.05, -1.4</td>
<td>-</td>
<td>-</td>
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<tr>
<td>16</td>
<td>Adenyate kinase isoenzyme 1</td>
<td>21,362</td>
<td>13959400</td>
<td>281</td>
<td>2</td>
<td>46%</td>
<td>&lt;0.05, -1.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stress Response &amp; Iron Homeostasis</td>
<td>17</td>
<td>60kDa heat shock protein, mt</td>
<td>60,917</td>
<td>51702230</td>
<td>1550</td>
<td>12</td>
<td>47%</td>
<td>-</td>
<td>-</td>
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<tr>
<td>18</td>
<td>Alddehyde dehydrogenase, mt</td>
<td>56,502</td>
<td>1352250</td>
<td>280</td>
<td>2</td>
<td>23%</td>
<td>&lt;0.05, -1.3</td>
<td>-</td>
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</tr>
<tr>
<td>19</td>
<td>Alpha crystallin B chain</td>
<td>20,056</td>
<td>6166129</td>
<td>761</td>
<td>7</td>
<td>65%</td>
<td>&lt;0.01, +1.3</td>
<td>-</td>
<td>&lt;0.01, +1.5</td>
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<tr>
<td>20</td>
<td>Myoglobin</td>
<td>17,059</td>
<td>1276767</td>
<td>203</td>
<td>2</td>
<td>38%</td>
<td>&lt;0.01, -1.7</td>
<td>-</td>
<td>-</td>
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<tr>
<td>21</td>
<td>Serum transferrin</td>
<td>76,674</td>
<td>21363012</td>
<td>121</td>
<td>1</td>
<td>17%</td>
<td>-</td>
<td>&lt;0.05, -1.3</td>
<td>-</td>
</tr>
<tr>
<td>Cross-Bridge &amp; Sarcoplasmic reticulum</td>
<td>22</td>
<td>Actin α</td>
<td>42,024</td>
<td>62287933</td>
<td>954</td>
<td>8</td>
<td>57%</td>
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<td>&lt;0.01, +1.4</td>
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<tr>
<td>23</td>
<td>Calreticulin</td>
<td>47,965</td>
<td>117502</td>
<td>366</td>
<td>3</td>
<td>43%</td>
<td>&lt;0.01, -1.4</td>
<td>&lt;0.01, +1.4</td>
<td>-</td>
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</tbody>
</table>
Table 2: MS Protein Spot Identifications from Sternohyoid Muscle

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein</th>
<th>Mw (Da)</th>
<th>GI number</th>
<th>Mascot score</th>
<th>MS/MS Matched Peptides</th>
<th>Sequence Coverage</th>
<th>Carbonyl p-value, fold</th>
<th>Free thiol p-value, fold</th>
<th>Expression p-value, fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytochrome bc-1 complex subunit 1</td>
<td>52,819</td>
<td>341941780</td>
<td>430</td>
<td>6</td>
<td>26%</td>
<td>-</td>
<td>&lt;0.01,-1.4</td>
<td>&lt;0.001,-2</td>
</tr>
<tr>
<td>2</td>
<td>ATP Synthase subunit α</td>
<td>59,716</td>
<td>416677</td>
<td>997</td>
<td>6</td>
<td>53%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01,1.5</td>
</tr>
<tr>
<td>3</td>
<td>Aconitase hydratase</td>
<td>85,410</td>
<td>60391212</td>
<td>1210</td>
<td>11</td>
<td>41%</td>
<td>&lt;0.01,-1.4</td>
<td>&lt;0.001,-2</td>
<td>&lt;0.01,-2.6</td>
</tr>
<tr>
<td>4</td>
<td>2-oxo-glutarate dehydrogenase, mt</td>
<td>116,375</td>
<td>146345472</td>
<td>477</td>
<td>4</td>
<td>22%</td>
<td>-</td>
<td>&lt;0.01,-2.4</td>
<td>&lt;0.01,-2.8</td>
</tr>
<tr>
<td>5</td>
<td>Fructose bis-phosphate aldolase A</td>
<td>39,331</td>
<td>113607</td>
<td>588</td>
<td>3</td>
<td>70%</td>
<td>-</td>
<td>&lt;0.01,-2.9</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>35,787</td>
<td>120702</td>
<td>531</td>
<td>4</td>
<td>48%</td>
<td>-</td>
<td>&lt;0.01,-1.6</td>
<td>&lt;0.001,-2</td>
</tr>
<tr>
<td>7</td>
<td>Phosphoglycerate Kinase 1</td>
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<td>534</td>
<td>4</td>
<td>45%</td>
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<td>-</td>
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<td>8</td>
<td>Phosphoglycerate Mutase 2</td>
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<td>6093745</td>
<td>1010</td>
<td>9</td>
<td>57%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01,1.7</td>
</tr>
<tr>
<td>9</td>
<td>Pyruvate kinase isoforms M1/M2</td>
<td>57,305</td>
<td>146345448</td>
<td>837</td>
<td>6</td>
<td>53%</td>
<td>&lt;0.01,-1.2</td>
<td>-</td>
<td>&lt;0.01,1.3</td>
</tr>
<tr>
<td>10</td>
<td>Glycerol-3-phosphate dehydrogenase [NAD(+)]</td>
<td>37,548</td>
<td>121557</td>
<td>412</td>
<td>3</td>
<td>48%</td>
<td>-</td>
<td>&lt;0.01,-2</td>
<td>&lt;0.01,-2.6</td>
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<tr>
<td>11</td>
<td>Creatine kinase m-type</td>
<td>43,014</td>
<td>124056470</td>
<td>784</td>
<td>7</td>
<td>42%</td>
<td>&lt;0.01,-1.2</td>
<td>-</td>
<td>&lt;0.01,-1.2</td>
</tr>
<tr>
<td>12</td>
<td>Glycogen Phosphorylase</td>
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<td>1450</td>
<td>13</td>
<td>47%</td>
<td>&lt;0.01,-1.3</td>
<td>-</td>
<td>&lt;0.01,4.1</td>
</tr>
<tr>
<td>13</td>
<td>90kDa heat shock protein</td>
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<td>6</td>
<td>31%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01,-1.3</td>
</tr>
<tr>
<td>14</td>
<td>Alpha crystallin B chain</td>
<td>20,056</td>
<td>6166129</td>
<td>761</td>
<td>7</td>
<td>65%</td>
<td>&lt;0.01,-1.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>Myoglobin</td>
<td>17,059</td>
<td>127676</td>
<td>923</td>
<td>7</td>
<td>43%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01,-1.7</td>
</tr>
<tr>
<td>16</td>
<td>Serotransferrin</td>
<td>76,674</td>
<td>21363012</td>
<td>121</td>
<td>1</td>
<td>17%</td>
<td>&lt;0.01,-1.4</td>
<td>&lt;0.01,1.8</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Carbonic Anhydrase III</td>
<td>29,348</td>
<td>30581036</td>
<td>966</td>
<td>8</td>
<td>71%</td>
<td>&lt;0.01,-1.2</td>
<td>&lt;0.01,-2.7</td>
<td>&lt;0.01,1.9</td>
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<tr>
<td>18</td>
<td>Albumin</td>
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<td>5915682</td>
<td>1340</td>
<td>11</td>
<td>53%</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01,-1.8</td>
</tr>
<tr>
<td>19</td>
<td>Calsequestrin</td>
<td>46,420</td>
<td>341940315</td>
<td>543</td>
<td>4</td>
<td>19%</td>
<td>&lt;0.01,-1.5</td>
<td>-</td>
<td>&lt;0.01,-2.3</td>
</tr>
</tbody>
</table>
3.2.3 Section C: Antioxidants

Total basal GSH content was higher in the diaphragm control compared to sternohyoid (Fig. 3.9) but this would be expected owing to the greater oxidative capacity of the diaphragm muscle. Six weeks of CH exposure increased diaphragm total GSH content (Fig. 3.9A) approaching statistical significance (5.40 ± 0.8 vs. 9.09 ± 1.76 mean ± SEM nmol/µg protein; control vs. CH; diaphragm tGSH after six weeks of CH; n=8 per group; p=0.07) and significantly increased sternohyoid total GSH content (3.02 ± 0.62 vs. 8.13 ± 1.9 mean ± SEM nmol/µg protein; control vs. CH; sternohyoid tGSH after six weeks of CH; n=7-8 per group; p<0.05) (Fig. 3.9B).

Diaphragm catalase activity (Fig. 3.10) is significantly increased after one, three and six weeks of CH compared to control (281.65 ± 21.89 vs. 512.73 ± 39.93***, 250.19 ± 26.12 vs. 363.10 ± 28.88*, 300.38 ± 42.55 vs. 565.65 ± 112.93* mean ± SEM Units/mg protein; control vs. CH; diaphragm catalase activity after one, three and six weeks of CH respectively; n=8 per group; *p<0.05, ***p<0.001).

---

**Figure 3.9:** Diaphragm and sternohyoid total GSH content after six weeks of sustained hypoxia compared to normoxic control. Diaphragm (A) and sternohyoid (B) total GSH (mean ± SEM) expressed nmol/µg protein; n=7-8 per group; *p<0.05; Student’s t test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Figure 3.10: Diaphragm catalase activity after one, three, and six weeks of sustained hypoxia compared to normoxic control. Diaphragm catalase activity (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as Units/mg protein; n=8 per group; *p<0.05, ***p<0.001; Student’s t test or Mann-Whitney test as appropriate; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO2 = 0.1).

3.3 Chapter Discussion

These results support the hypotheses of 1) differential and temporal changes in respiratory and limb muscle protein carbonyl and free thiol content; 2) key proteins being redox modified; and 3) of increased antioxidant defences following CH exposure.

3.3.1 Total Protein Carbonyl and Free Thiol Content

Protein oxidation in diaphragm muscle is progressive. It is conceivable that the diaphragm becomes more susceptible to injury with progressive hypoxia exposure just as diaphragm injury is a cause of death in COPD patients. Similar changes are observed in the sternohyoid and to a lesser extent in limb muscles meaning hypoxia-induced protein redox remodelling is likely exacerbated by increased contractile activity in hypoxia. Differential protein oxidation in response to CH has previously been reported in human limb muscles following high altitude hypoxia exposure [96].
Increased protein free thiol content after one week, observed in all four muscles studied, is suggestive of an early antioxidant response independent of workload or fibre type composition although the greatest increases are observed in the soleus and diaphragm which have the greater complement of slow oxidative fibres. Regardless, this potential antioxidant response is likely an effect of hypoxia *per se*, especially when considered that these changes are witnessed before classic acclimation responses such as increased haematocrit. Within the respiratory and limb muscle groups, the diaphragm and soleus muscles present with greatest fold decreases when comparing the effects of CH after one week with that of six weeks indicating an inherent susceptibility of the slow oxidative fibre phenotype. The temporal fold change differences in protein carbonylation are further indicative of this. This makes physiological sense given that 1) the slow oxidative muscles rely more on mitochondria to produce required ATP and would be subjected to greater electron leak and subsequent superoxide formation and protein oxidation if this process is disrupted; but also have a faster response to stress that is also greater in magnitude; and 2) greater activity would produce greater amounts of ROS; thus, the greater complement of slow oxidative fibre types in a given muscle and increased muscle activity places the muscle at greater risk of protein oxidation.

Soleus and EDL control values for protein carbonyl and free thiol content are comparatively more in concert with what would be expected from slow oxidative and fast glycolytic fibre type muscles than the diaphragm and sternohyoid muscles comparatively (i.e. more protein carbonyl content and less free thiol content in the slow oxidative muscles compared to fast glycolytic muscles). The respiratory muscles, in particular the diaphragm, are clearly unique in this regard. The soleus has a greater density of protein free thiol groups in control samples in comparison to the EDL and both respiratory muscles which may also be reflective of its fibre type distribution.

Curiously, after three weeks of CH, the increase in diaphragm protein free thiol content is substantially greater than in the other muscles studied. This may confer a greater degree of protein thiol protection and/or be indicative of greater protein
turnover in the diaphragm in and around this time point. If endurance capacity is correlated with protein free thiol content and ROS production is the principle reason behind diminishing muscle performance \textit{ex vivo}, this change may give the diaphragm an advantage over other muscles in terms of muscle endurance at this time point (or equivalent in other rodents), in hypoxia, while the muscle is being studied \textit{ex-vivo}.

An interesting side-note: oxidative stress does not appear to affect protein structure and function in the naked-mole rat who lives underground in hypoxic conditions [223].

3.3.2 Redox Proteomics

Our aim for this study was to identify what proteins are subject to change by using a redox proteomics approach. We hypothesized that proteins key to muscle function will be redox modified in the respiratory muscles after six weeks of CH. Given that muscle metabolism, homeostasis and function are inextricably linked, and that specific oxidation of several of the proteins we have identified have been shown by other groups to have their catalytic activity or functional capability modified by oxidation (e.g. actin; discussed below); the results of this study support our hypothesis.

Curiously, although we observe extensive global changes to protein carbonyl and free thiol content in both respiratory muscles, there is an apparent specificity given the somewhat similar changes observed in the respective muscles despite their intrinsic molecular differences. This draws attention to a potential role of redox signalling in respiratory muscles in animals exposed to CH. Indeed, global phosphatase activity in muscle is reported as altered with redox modulation [224]. However, whilst many of the proteome changes are similar between diaphragm (table 3.1) and sternohyoid (table 3.2) e.g. it is clear that metabolic proteins are extensively modified in both, it is also evidently clear that there is a unique redox fingerprint for both muscles after six weeks of CH, potentially a result of proteome composition i.e. the sternohyoid consists of a greater relative abundance of glycolysis enzymes to TCA cycle enzymes in comparison to the diaphragm.
2-oxo-glutarate dehydrogenase, actin, and the electron transport chain (ETC) proteins (when taken together) present with greatest remodelling in the diaphragm and 2-oxo-glutarate dehydrogenase, glycogen phosphorylase, and carbonic anhydrase III (CAIII) present with greatest remodelling in the sternohyoid. Redox-remodelling of 2-oxo-glutarate dehydrogenase, a TCA cycle enzyme, is likely to limit ROS production by both its own oxidoreductase activity and by mitochondria [139]. CAIII rehydrates CO₂; a potential role for this enzyme in lactate shuttling through monocarboxylate transporters and other transporters has also been suggested [225]. Interestingly, redox remodelling of this protein is also observed in respiratory muscle of COPD patients [153] and CAIII has been shown to be significantly up-regulated after exercise in humans, but only in hypoxia [226]. Furthermore, CAIII has been shown to have important anti-apoptotic effects in oxidatively stressed cells [227] and may confer protection for the upper-airway dilator muscle in hypoxia as both an antioxidant and pH regulator. Glycogen phosphorylase regulates glycogen breakdown, and is increased more than 4-fold after CH exposure in the sternohyoid. Of interest, no change is observed for this enzyme in human vastus lateralis with ascent to high altitude [228], potentially indicative of selective importance to the sternohyoid. The large up-regulation would be expected to increase carbohydrate substrate availability for energy production, required to maintain the increased continuous rhythmic function in the sternohyoid during CH-induced hyperactivity. Furthermore, the ATP-producing capacity of the “second” part of the glycolytic pathway may be enhanced through the increased expression of phosphoglycerate kinase, phosphoglycerate mutase and the pyruvate kinase isozymes. This enhanced step 2 of glycolysis, alongside increased glycogen phosphorylase content is potentially fundamental to the maintenance of enhanced sternohyoid contractile activity in hypoxia. In comparison, the enduring phenotype of the diaphragm is perhaps best suited to increased expression and reduction/turnover of ETC proteins. Despite reduced mitochondria content, an improved working capacity of individual mitochondria in the murine diaphragm after CH exposure has been suggested to be due to decreased UCP3 expression [67] and increased expression of complexes IV and V. In line with this finding, we observe increases in cytochrome bc-1 complex subunit 1 and ATP Synthase subunit β as well as increased free thiol content in ATP Synthase subunit α and a subunit of the electron transfer flavoprotein in the CH
diaphragm. The increased free thiol content is potentially due to increased turnover of protein or folding changes as there is also an increase in 60kDa heat shock protein (HSP) content.

Redox modifications at the level of the cross-bridge can directly facilitate functional modifications between muscle fibres of the same type [160,186,229–234]. Figure 3.12 (unpublished pilot study data from Persson, M., 2013; shown here with permission) illustrates how actin oxidation can affect cross-bridge sliding velocity in-vitro. This is potentially very important given the lack of fibre type change in respiratory muscles in animal models of CH [22,65,67]. When there is an increase in the oxidising environment in muscle, it is likely that the cross-bridge will be affected as a result of the abundance and size of these proteins [187,204,235–237]. Oxidative damage to sarcomeric proteins is observed after mitochondrial dysfunction in the heart [237]. However, it is difficult to observe actomyosin and auxiliary protein signals during proteomic investigations [238]. Despite this difficulty, we observe oxidation of actin-α alongside an expression increase in the diaphragm muscle, and also oxidation of α-crystallin B chain and CK m-type in both diaphragm and sternohyoid muscles. Actin is a critical component for cross-bridge formation and muscle contraction, CK m-type supplies ATP for mATPase activity and α-crystallin B chain is a sarcomere localised chaperone protein [239]. Actin oxidation is also observed in several disease settings including COPD, Alzheimer’s disease and cardiac ischemia [153–155].
Figure 3.12 In-vitro motility assay for control and oxidised (H$_2$O$_2$) treated F-actin (unpublished pilot study data from Persson, M., 2013 and shown here with permission). (A) Sliding velocity data of actin over myosin. The number of filament paths for velocity measurements in each run is 2-6. (B) Fraction of motile filaments in the assay. The images on the bottom are micrographs of untreated and H$_2$O$_2$ treated F-actin (left and right respectively). The sliding velocities for the oxidized F-actin were reduced compared to the untreated F-actin (7.750 ± 0.2902 vs. 4.347 ± 0.9561; untreated vs. oxidised; mean ± SEM; n=3 per group (n is the number of different flow cells used i.e. different salinization batches)). Furthermore, the fraction of motile filaments was also reduced. Both untreated and H$_2$O$_2$-treated actin filaments had remained in their filamentous form and phalloidin labelling was not affected by the oxidation. In one control experiment, normal untreated F-actin labelled with Alexa-488-phalloidin was incubated in the same flow cell as Rhodamine-phalloidin labelled H$_2$O$_2$-treated F-actin. The normal F-actin exhibit good motility while the oxidized F-actin (H$_2$O$_2$-treated) did not. The sliding velocity and fraction motile filaments from the control experiments are included. This shows that the myosin heads are not affected by any possible difference in actin solutions e.g., H$_2$O$_2$ residuals. The actin filament lengths influence on the sliding velocity is not taken into consideration in the data presented here.
There is extensive redox remodelling of metabolic proteins in both muscles. The localisation of these proteins coupled with remodelling of the mitochondrial respiratory chain and stress responsive proteins leads us to believe the mitochondria are the source of the remodelling stress. Diminishing electron transfer between respiratory chain complexes assists the upstream modules becoming predisposed to autoxidation. This is supported with the findings of mitochondrial remodelling and ‘mitophagy’ in CH-murine diaphragm [67,80], in limb muscles of humans at high altitude [97], in COPD patient diaphragm muscle [165] and in COPD and hypoxaemic patient limb muscles [240,241].

CK, and aconitase are metabolic proteins susceptible to oxidation and this is observed in the present study. Oxidation of these enzymes correlates with negative regulation of their activities [153,204] and CK is oxidised in the diaphragm of COPD patients [153]. Aconitase is often measured as a marker of oxidative stress. CK deficiency is associated with deficiencies in burst activity of muscle [242] and this may have particular relevance to the sternohyoid from a functional perspective as this activity is required to reopen the airway when occlusions occur during sleep. There is less mitochondrial CK content and an increase in cytoplasmic CK oxidation in the six week CH diaphragm as well as less cytoplasmic CK content with increased oxidation in the sternohyoid. This, along with the observed oxidation of adenylate kinase in the diaphragm, will impact upon cytosolic adenosine monophosphate (AMP):ADP:ATP levels in these muscles, which will in turn affect AMP Kinase (K) activity – an important determinant of many cellular processes including the balance between energy production and utilisation. Indeed, hypoxia has previously been shown to regulate AMPK in a ROS dependent manner [243]. Further muscle specific regulation of metabolic enzymes includes aldolase and β-enolase which are significantly and differentially modified in diaphragm and sternohyoid muscles after six weeks of CH.

Carbonylation of α-crystallin B chain, a cross-bridge chaperone described as essential to myofilament maintenance [244], is observed in both muscles. Forms of CRYAB gene-linked myopathy can be suppressed by altering availability of reducing equivalents such as NADPH, supporting a role for ROS regulation [245].
Furthermore, α-crystallin B chain is described as having antioxidant-like properties [246]. Of note, strength training increases its expression [247]. Just as the maintenance of proper folding of cross-bridge proteins in working muscle is fundamental to function, α-crystallin B chain may be an important player downstream of ROS in the differential functional adaptations observed in respiratory and limb muscles [22,26,27,67,79].

Myoglobin is an oxygen binding protein with a ferrous core that is expressed in skeletal muscle and presents with decreased carbonyl content and expression in diaphragm and sternohyoid respectively which may be important in reducing the formation of the extremely reactive hydroxyl radical by decreasing Fenton reactions. Decreased myoglobin expression has been previously observed in skeletal muscle in response to hypoxia [122].

In addition to α-crystallin B chain, several stress response proteins involved in homeostasis maintenance and oxidant buffering present with redox/expression modifications including HSPs, serotransferrin, albumin, and aldehyde dehydrogenase. HSPs are correlated with skeletal muscle fibre type [248] and are involved in maintaining mitochondrial integrity [249], so their respective modulation may have a role in differential functional outcomes in respiratory and limb muscles. Small HSPs have been shown to modulate ROS, raise GSH levels, prevent apoptosis signalling derived from stressed mitochondria, and activate the proteasome [150,250,251] while it is also plausible that oxidative modification and/or expression changes of protein chaperones facilitates atrophy instead of re-folding [252]. Alterations to HSPs are likely to affect atrophy and thus function following CH.

GAPDH is carbonylated after six weeks of CH in the diaphragm muscle with no detectable change in its expression. While expression is decreased in the sternohyoid after CH, reductive redox remodelling is observed similar to glycogen phosphorylase which suggests lesser oxidation, protection, or both increased translation and ROS induced degradation occurring simultaneously albeit at different rates, – potentially as a result of HIF and ROS. GAPDH is a key metabolic switch protein. With oxidation, GAPDH relocates to the nucleus [253] and metabolic substrate flux is
directed toward the pentose phosphate pathway to increase reducing power of the cell by NAD(P)H formation [212,254,255].

Redox remodelling of fatty acid binding protein (FABP) and glycerol-3-phosphate dehydrogenase [NAD+] cytoplasmic along with the increased catalase activity are suggestive of a redox effect on fatty-acid utilisation – a change which is known to occur in cardiac tissue after CH [256]. In control conditions there is differential expression of lipid and carbohydrate metabolism genes within the respiratory muscles with the diaphragm favouring lipid metabolism in comparison to the sternohyoid [257].

Differential remodelling of Ca²⁺ buffering proteins and Ca²⁺ activated chaperones in the SR may be involved in affecting Ca²⁺ signalling in muscle. Muscle bundle twitch contractile kinetics are unaltered by CH [22] as are single fibre shortening velocities [70] so release and re-uptake are unlikely affected. Hypoxia has been observed to alter Ca²⁺ signalling in certain circumstances [70,122,184,243,258–260], and oxidised calreticulin and decreased expression of calsequestrin in diaphragm and sternohyoid muscles respectively may have a role in this [261,262].

3.3.3 Antioxidants
We measured a significant increase in catalase activity in the diaphragm that remained elevated after three and six weeks of exposure – evidence of an early antioxidant response (correlating with increased protein free thiols). There is also an increase in the total GSH content after six weeks of CH in both respiratory muscles, reaching statistical significance in the sternohyoid and approaching it in the diaphragm. Conflicting with these findings, it has been reported that hypoxia exposure decreases mitochondrial ROS scavenging proteins [97]. While carbonylation is indicative of structural damage, increased tGSH may contribute to decreased free thiol content in the respiratory muscles after six weeks of CH through formation of disulphide bridges with proteins (RS-SG) providing protection from ROS. With the data from the proteomic study highlighting extensive remodelling to mitochondrial and metabolic proteins and the increase in antioxidant capacity
reported here, we hypothesize that ROS from mitochondria are causing these redox changes and oxidative stress is not the result of a decrease in antioxidant status.

3.3.4 Conclusion
In conclusion, our findings show that CH induces differential redox remodelling of the murine respiratory and limb muscle proteome; proteins key to muscle function are affected in the diaphragm and sternohyoid, and antioxidant status is not compromised. Many protein carbonyl changes we observe are similar to those observed in human COPD diaphragm and LPS injected rat diaphragm tissue [72,204]. Mitochondria are the candidate source of stress following CH. Antioxidants may be useful as an adjunct therapeutic option in diseases where CH is featured, however selectivity is required as to the type of antioxidant and its target given the potential role for redox signalling in these muscles [224]. This study also highlights the potential of the redox proteomics approach [263] to be extended to other models of disease featuring oxidative/reductive stress.
Chapter 4: Metabolic adaptation in the diaphragm following chronic sustained hypoxia
4.1 Chapter Introduction

Mammals cannot survive prolonged periods of very severe hypoxia due to loss of ion homeostasis in the vital organs unless they have undergone graded acclimatisation [4,264,265]. Less oxygen results in perturbed cellular respiration, decreased ATP levels, slowdown of the Na\(^{+}/K^{+}\) pump, and ion imbalance. Cellular pH drops with lactic acid production, the cell swells and membranes become leaky. Maintaining ion homeostasis requires the use of energy dependent ion pumps and so metabolic regulation in hypoxic vital organs is imperative.

Skeletal muscle is considered amongst the most hypoxia-tolerant tissues [265] which is fascinating considering that skeletal muscle has high basal levels of mATPase activity as well as ATP-dependent ion pump activity [266]. These high ATPase activities accompanied with hypoxia should place tighter regulation on both ATP production and utilisation in this organ. Regulation of metabolism is arguably even more important in the diaphragm muscle during CH as the muscle is vital and more active. Increased ventilatory activity of the muscle will increase energy expenditure and demand through increased mATPase activity and increased H\(^{+}\) accumulation in the cytoplasm with subsequent requirement for removal. An increase in Na\(^{+}/K^{+}\) ATPase pump content has been observed in rat diaphragm exposed to six weeks of CH [22].

O\(_{2}\) is the ultimate electron acceptor in oxidative phosphorylation and required for ATP synthesis by this method, but hypoxia and HIF-1\(\alpha\) signalling is typically associated with a shift toward glycolytic/anaerobic metabolism [95,267,268]. However, predominant glycolytic metabolism is unlikely to be able to support continuous diaphragm activity. The hypoxic ventilatory response may be viewed as a training effect and this should increase oxidative metabolism under normal circumstances [269]. Given the functional phenotype of the diaphragm following CH, metabolic adaptations are likely to occur to sustain/maintain functional integrity.

Only a single study to date concerning metabolic enzyme activities in the diaphragm muscle of animal models of CH has been conducted [22] and no change was
observed. The study in question performed histochemistry on sections of rat respiratory muscle tissue followed by analysis of captured images to assess SDH activity. However, it has been reported that enzyme changes may be fibre/region specific within a muscle [87] and this is something we sought to explore further. [22]. Using the same tissue section images from [22], we measured SDH activity in individual fibres, plotted the results as a function of fibre cross sectional area and separated them into the most spatially distinct clusters using K-means cluster analysis in order to identify shifts in cluster fibre proportions or cluster centroids. We hypothesized that there would be an increase in the proportion of fibres in the higher SDH activity clusters because an increase in diaphragm activity and improved endurance is associated with increased oxidative capacity.

It would be remiss to assume that the behaviour of a single protein is representative of an organelle, process, or pathway as a whole, especially when there is more than one stimulus and these stimuli being known to act upon different proteins (hypoxia vs. increased activity vs. redox stress) [270]. We have described redox remodelling of respiratory muscle metabolic enzymes in CH in Chapter 3, similar to COPD where redox modified metabolic enzyme activity changes occur [153]. The potential exists for significant physiological changes to metabolic enzyme activities in hypoxia through this mechanism [204] e.g. aconitase enzyme activity is correlated with oxidative stress [102,271,272]. In times of oxidative stress, it is also known that metabolic flux re-routing occurs to increase substrate availability for the pentose phosphate pathway to increase reducing equivalents for antioxidant usage [254]. We have also shown increased catalase activity in the diaphragm muscle after one, three and six weeks of CH (Chapter 3) and catalase is a necessary enzyme to decompose H$_2$O$_2$ that is produced in peroxisomes by β-oxidation, along with redox remodelling of proteins involved in lipid metabolism – FABP and G3PD. Indeed, β-oxidation would provide an alternate route of NADH and FADH to the respiratory chain complexes whilst facilitating NADPH production for antioxidant activity [273]. The second aim of this chapter was to measure metabolic activities of enzymes observed to undergo redox remodelling (chapter 3), as well as enzymes located at carbohydrate derivative flux gating points in respiratory and limb muscles after CH exposure (Fig. 4.1). We hypothesized that enzyme activity changes would be
indicative of favouring substrate flux into the pentose phosphate pathway, with decreased activity of glycolytic enzymes, as well as both decreased aconitase enzyme and CK enzyme activities for the diaphragm. Furthermore, we speculated that differential remodelling of metabolic enzymes would be observed in respiratory and limb muscles.

In addition, we assessed HIF-1α content in the diaphragm and sternohyoid muscle after six weeks of CH ± chronic antioxidant supplementation. HIF-1α is a subunit of the HIF transcription factor complex that is constitutively expressed and degraded in normoxic conditions but degradation is prevented under hypoxic conditions. It promotes transcription of the cellular defensive response to hypoxia by forming a complex with the HIF-β subunit and consequent up-regulation of erythropoietin and haematocrit, glycolytic enzymes which produce ATP anaerobically etc. [95,267,274]. We know HIF-1α is basally and differentially expressed in skeletal muscle [7,13] and is involved in fast fibre type gene expression whereas the competing HIF-2α subunit is implicated in driving the slow fibre phenotype [14,15,144]. As discussed in chapter 1 and chapter 3, we consider the mitochondria as the primary site of both sensing hypoxia in the diaphragm and responding in the form of ROS production and altered redox remodelling. There is evidence to suggest that the transcription factor HIF-1α can be also modulated by ROS [148,164,275–277] with some evidence implicating oxygen sensing at the mitochondria followed by ROS production and a role for the energy and stress sensors AMPK and MAPK proteins. One week of high altitude exposure has no effect on HIF-1α levels in human vastus lateralis [97] while two weeks of CH increased HIF-1α in rat gastrocnemius [278]. Training in hypoxia also induces HIF-1α mRNA transcription [279]. Potentially longer durations of hypoxia with/without the requirement of redox signalling are required for HIF-1α activation in diaphragm muscle. We know HIF-1α is activated to increase red blood production which occurs in our model. Thus, HIF may play a role in diaphragm adaptation to hypoxia and redox change and should duly be quantified.
Figure 4.1: Overview of metabolism pathways in skeletal muscle. Enzymes whose activity is measured in this thesis are highlighted with a green background. G6PD = glucose-6-phosphate dehydrogenase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; G3PD = glycerol-3-phosphate dehydrogenase; LDH = lactate dehydrogenase; SDH = succinate dehydrogenase; CK = creatine kinase.
4.2 Chapter Results

4.2.1 Section A: SDH activity at the fibre level

The difference in SDH activity and the mitochondrial population (and ultimately fibre type composition) between the diaphragm and sternohyoid muscles is evident from the muscle section stained images for SDH activity in Fig. 4.2. Different structural and metabolic profiles may partly explain differential adaptations in these muscles. These differences can also be seen in Fig. 4.3 and Fig. 4.4 with the proportions of fibres at particular cross-sectional areas SDH activities distinctly different in diaphragm and sternohyoid muscles. The SDH activity correlation with fibre size can also be seen in Fig. 4.3 and Fig. 4.4.

K-means cluster analysis is an unbiased way of separating the fibres in the most spatially distinct fibre clusters according to both SDH and fibre cross sectional area. Log scales are used to prevent skew in the large data sets.

These data reveal no statistically significant differences in respective cluster centroids or the proportion of fibres associated with each cluster (Fig. 4.4) when normoxic and CH diaphragm and sternohyoid muscles were compared (29.8 ± 3.16 vs. 29.5 ± 2.82, 34.83 ± 1.3 vs. 37.17 ± 3.0, 38.67 ± 3.22 vs. 33.5 ± 2.72 mean ± SEM percentage proportion of fibres in diaphragm clusters one, two, and three respectively; control vs. hypoxia; n=6 per group; Student’s two-tailed unpaired t test) (28.0 ± 3.66 vs. 28.0 ± 1.92, 29.8 ± 2.29 vs. 24.2 ± 1.56, 42.2 ± 4.99 vs. 48.0 ± 3.2 mean ± SEM percentage proportion of fibres in sternohyoid clusters one, two, and three respectively; control vs. hypoxia; n=5 per group). In sternohyoid cluster 2, there is a decrease in the percentage proportion of fibres toward cluster 3 but this does not reach statistical significance (p=0.07).
Figure 4.2: 10µm transverse section of rat diaphragm (top) and sternohyoid (bottom) muscle stained for SDH activity. Oxidative fibre types are intensely stained compared to the pale glycolytic fibres. Scale represents = 200µm
Figure 4.3: Representative area-density plots for diaphragm (top) and sternohyoid (bottom). Using these data, K-means cluster analysis centroids for three clusters were determined for each plot with relative proportion of fibres in each defined cluster (see Fig. 4.4 below).
Figure 4.4: Scatterplots of cluster centroids for diaphragm (top) and sternohyoid (bottom) in CH-treated (blue) and normoxic (red) rats. There was no significant difference in respective cluster centroids or the proportion of fibres associated with each cluster when normoxic and CH muscles were compared.
4.2.2 Section B: Metabolic enzyme activities and HIF-1α

Aconitase is the 2nd enzyme of the TCA cycle and known to have its catalytic activity decreased in times of oxidative stress owing to its catalytic site iron-sulphur cluster. As shown in Fig. 4.5A, activity is significantly decreased after three and six weeks of CH while no change is observed after one week (0.397 ± 0.05 vs. 0.34 ± 0.02, 0.43 ± 0.04 vs. 0.147 ± 0.02**, 0.455 ± 0.009 vs. 0.243 ± 0.042* mean ± SEM Units/mg protein; control vs. CH; diaphragm aconitase activity after one, three, and six weeks of CH respectively; n= 4-6 per group; *p<0.05, **p<0.01).

CK, a mitochondrial and cytoplasmic enzyme that facilitates having a readily available but short-term supply of ATP when required, has an apparent bi-phasic response in hypoxia. CK activity is increased after one week of CH compared to control (p=0.059), but has significantly decreased activity after three and six weeks of CH (2.58 ± 0.14 vs. 3.42 ± 0.35#, 2.5 ± 0.37 vs. 1.31 ± 0.08*, 3.12 ± 0.28 vs. 1.89 ± 0.29* mean ± SEM Units/mg protein; control vs. CH; diaphragm CK activity after one, three, and six weeks of CH respectively; n= 4-6 per group; #p=0.059 *p<0.05), as is illustrated in Fig. 4.5B.

Aldolase is the 3rd enzyme of the glycolysis pathway. Fig. 4.6A shows significantly decreased activity in the diaphragm after one and three weeks of CH and decreased activity (p=0.08) after six weeks of CH (0.104 ± 0.01 vs. 0.07 ± 0.005*, 0.15 ± 0.01 vs. 0.062 ± 0.003***, 0.13 ± 0.01 vs. 0.09 ± 0.01 mean ± SEM Units/mg protein; control vs. CH; diaphragm aldolase activity after one, three and six weeks of CH respectively; n=5-7 per group; *p<0.05, ***p<0.001).

G3PD is an important link between carbohydrate and lipid metabolism directing substrate for lipid metabolism, gluconeogenesis or the mitochondrial glycerol-phosphate shuttle. Interestingly, despite being oxidatively modified (Chapter 3), G3PD activity is significantly increased after one, three, and six weeks of CH as illustrated in Fig. 4.6B (0.57 ± 0.02 vs.1.09 ± 0.11***, 0.71 ± 0.07 vs. 1.24 ± 0.17*, 0.48 ± 0.05 vs. 1.04 ± 0.15** mean ± SEM Units/mg protein; control vs. CH; diaphragm G3PD after one, three and six weeks of CH respectively; n=7-8 per group; *p<0.05, **p<0.01, ***p<0.001).
Figure 4.5: Diaphragm aconitase and creatine kinase activity after one, three, and six weeks of sustained hypoxia compared to normoxic controls. (A) Diaphragm aconitase activity (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as Units/mg protein; n= 4-6 per group; (B) Diaphragm creatine kinase activity (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as Units/mg protein; n= 4-6 per group; *p<0.05, **p<0.01, ns = not significant, Student’s t test and Mann-Whitney test as appropriate; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1).
Figure 4.6: Diaphragm aldolase and G3PD activity after one, three, and six weeks of sustained hypoxia compared to normoxic controls. (A) Diaphragm aldolase activity (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as Units/mg protein; n= 5-7 per group; (B) Diaphragm G3PD activity (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as Units/mg protein; n= 7-8 per group; *p<0.05, **p<0.01, ***p<0.001; Student’s t test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
G6PD is a metabolic enzyme that shuttles glucose-6-phosphate into the pentose phosphate pathway for NADPH production. G6PD activity, as shown in Fig. 4.7A and contrary to our hypothesis, is unchanged after one week of CH and significantly decreased after three and six weeks of CH (3.27 ± 0.35 vs. 2.88 ± 0.24, 3.01 ± 0.39 vs. 1.54 ± 0.25**, 3.57 ± 0.60 vs. 1.15 ± 0.1274** mean ± SEM Units/mg protein; control vs. CH; diaphragm G6PD activity after one, three and six weeks of CH respectively; n=7-8 per group; **p<0.01).

LDH, which converts pyruvate to lactate in anaerobic respiration, follows a similar pattern to G6PD of unchanged activity after one week of CH and significantly decreased activity after three and six weeks of CH as illustrated in Fig. 4.7B (0.63 ± 0.034 vs. 0.586 ± 0.055, 0.73 ± 0.27 vs. 0.349 ± 0.061, 0.756 ± 0.099 vs. 0.38 ± 0.088* mean ± SEM Units/mg protein; control vs. CH; diaphragm LDH activity after one, three and six weeks of CH respectively; n=6-7 per group; *p<0.05).

GAPDH is a key enzyme of the glycolytic pathway that we observe as oxidised in the diaphragm after six weeks of CH. As can be seen in Fig. 4.8A, GAPDH activity is increased (p=0.08) after one week of CH but is significantly decreased after three weeks of CH (0.013 ± 0.002 vs. 0.020 ± 0.002, 0.015 ± 0.0012 vs. 0.010 ± 0.0016* mean ± SEM Units/mg protein; control vs. CH; diaphragm GAPDH activity after one and three weeks of CH respectively; n=5-7 per group; *p<0.01). Note: Due to muscle tissue sample volume limitations, GAPDH activity at the six week time point could not be measured.

Biphasic changes in GAPDH activity in the sternohyoid muscle after one and three weeks of CH are featured in Fig. 4.8B. GAPDH activity is significantly increased after one week of CH but significantly decreased after three weeks of CH (0.02 ± 0.001 vs. 0.023 ± 0.003*, 0.026 ± 0.001 vs. 0.018 ± 0.001** mean ± Units/mg protein; SEM; control vs. CH; sternohyoid GAPDH activity after one and three weeks of CH respectively; n=6-7 per group; *p<0.05. **p<0.01). Note: Due to muscle tissue sample volume limitations, GAPDH activity at the six week time point could not be measured.
Figure 4.7: Diaphragm G6PD activity and LDH activity after one, three, and six weeks of sustained hypoxia compared to normoxic controls. (A) Diaphragm G6PD activity (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as Units/mg protein; n= 7-8 per group; (B) Diaphragm LDH (mean ± SEM) after one, three, and six weeks of sustained hypoxia expressed as Units/mg protein; n= 6-7 per group; **p<0.01, ns = not significant, Student’s t test. Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Figure 4.8: Diaphragm and sternohyoid GAPDH activity after one and three weeks of sustained hypoxia compared to normoxic controls. (A) Diaphragm GAPDH activity (mean ± SEM) after one and three of sustained hypoxia expressed as Units/mg protein; n= 5-7 per group; (B) Sternohyoid GAPDH activity (mean ± SEM) after one and three weeks of sustained hypoxia expressed as Units/mg protein; n= 6-7 per group; *p<0.05, **p<0.01; Student’s t test and Mann-Whitney test as appropriate; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1).
GAPDH activity in EDL (Fig. 4.9A) is increased after one and three weeks of CH compared to controls (0.02 ± 0.001 vs. 0.028 ± 0.001*, 0.018 ± 0.001 vs. 0.025 ± 0.002* mean ± SEM Units/mg protein; control vs. CH; EDL GAPDH activity after one and three weeks of CH respectively; n=6-8 per group; *p<0.05, **p<0.01). Note: Due to muscle tissue sample volume limitations, GAPDH activity at the six week time point could not be measured.

Similar to EDL muscle, there are significant increases in GAPDH activity in the soleus (Fig. 4.9B) after one and three weeks of CH (0.015 ± 0.002 vs. 0.021 ± 0.001*, 0.013 ± 0.002 vs. 0.025 ± 0.005* mean ± SEM Units/mg protein; control vs. CH; soleus GAPDH activity after one and three weeks of CH respectively; n=7-8 per group; *p<0.05, **p<0.01). Note: Due to muscle tissue sample volume limitations, GAPDH activity at the six week time point could not be measured.

LDH activity in the sternohyoid (Fig. 4.10A) is significantly increased after one week of CH while there is no change observed after three weeks of CH compared to controls (0.598 ± 0.125 vs. 0.938 ± 0.099*, 0.644 ± 0.111 vs. 0.438 ± 0.041 Units/mg protein; mean ± SEM; control vs. CH; sternohyoid LDH activity after one and three weeks of CH respectively; n=5-8 per group; *p<0.05). Note: Due to muscle tissue sample volume limitations, LDH activity at the six week time point could not be measured.

There is no change LDH activity in the EDL (Fig. 4.10B) after one or three weeks of CH (0.714 ± 0.033 vs. 0.683 ± 0.034, 0.865 ± 0.151 vs. 0.712 ± 0.177 mean ± SEM Units/mg protein; control vs. CH; EDL LDH activity after one and three weeks of CH respectively; n=6-7 per group). Note: Due to muscle tissue sample volume limitations, LDH activity at the six week time point could not be measured.

Similarly, there is no change in soleus LDH activity after one week of CH compared to control (0.281 ± 0.027 vs. 0.299 ± 0.026 mean ± SEM Units/mg protein; control vs. CH; soleus LDH activity after one week of CH; n=7 per group). Note: Due to muscle tissue sample volume limitations, LDH activity at the three and six week time points could not be measured.
Figure 4.9: EDL and soleus GAPDH activity after one and three weeks of sustained hypoxia compared to normoxic controls. (A) EDL GAPDH activity (mean ± SEM) after one and three weeks of sustained hypoxia expressed as Units/mg protein; n= 6-8 per group; (B) Soleus GAPDH activity (mean ± SEM) after one and three weeks of sustained hypoxia expressed as Units/mg protein; n= 7-8 per group; *p<0.05, **p<0.01; Student’s t test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Figure 4.10: Sternohyoid, EDL and soleus LDH activity after one and three weeks of sustained hypoxia compared to normoxic controls. (A) Sternohyoid LDH activity (mean ± SEM) after one and three weeks of sustained hypoxia expressed as Units/mg protein; n= 5-8 per group; (B) EDL LDH activity (mean ± SEM) after one and three of sustained hypoxia expressed as Units/mg protein; n= 6-7 per group; (C) Soleus LDH activity (mean ± SEM) after one week of sustained hypoxia expressed as Units/mg protein; n= 7 per group; *p<0.05, ns = not significant; Student’s t test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
As illustrated in Fig. 4.11A, six weeks of CH significantly increased HIF-1α content in diaphragm muscle compared to control and this was attenuated by chronic antioxidant supplementation with either tempol or NAC (1.714 ± 0.58 vs. 2.635 ± 0.136*** vs. 1.557 ± 0.09### vs. 1.926 ± 0.112### mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; diaphragm HIF-1α content after six weeks of CH; n=5-8 per group; One-way ANOVA: p<0.001; ***p<0.001 vs. control, ###p<0.001 vs. hypoxia, Tukey’s multiple comparison test).

In contrast to diaphragm, no significant changes were observed to HIF-1α content in sternohyoid muscle compared to controls (2.07 ± 0.22 vs. 2.279 ± 0.239 vs. 1.946 ± 0.191 vs. 1.956 ± 0.154 mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; sternohyoid HIF-1α content after six weeks of CH; n=5-8 per group).
Figure 4.11: Diaphragm and sternohyoid HIF1-α content after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Diaphragm HIF1-α content mean ± SEM Units/mg protein; n= 5-8 per group; (B) Sternohyoid HIF1-α content mean ± SEM Units/mg protein; n= 5-7 per group; ***p<0.001 vs. control, ###p<0.001 vs. hypoxia Tukey’s multiple comparison test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); RLU = relative luminescence units.
4.3 Chapter Discussion

The main findings of this chapter are 1) SDH activity does not change at a fibre level in rat diaphragm following CH; 2) redox remodelling in the mouse diaphragm is associated with decreased aconitase, aldolase, and CK activities; 3) there may be an increased reliance on fatty acid metabolism or the glycerol-phosphate shuttle in the CH diaphragm; 4) differential muscle metabolic enzyme activity remodelling is observed in respiratory and in limb muscles; and 5) HIF-1α content is significantly increased in the diaphragm following six weeks of CH and is attenuated by antioxidant supplementation.

4.3.1 SDH activity at the fibre level

There is a trend towards a significant decrease in proportion of fibres in the 2\textsuperscript{nd} hypoxic sternohyoid cluster compared to control. The shift is in favour of decreased oxidative capacity as there is an increased mean proportion of fibres in the 3\textsuperscript{rd} cluster whose centroid coordinates are of lower SDH activity and higher FCSA. Hypertrophy is observed in the fast glycolytic fibres of rat sternohyoid following CH [22] so this trend potentially follows that while FCSA increases the SDH content and thus mitochondrial content does not. No change is observed in the diaphragm despite fibre atrophy of the slow oxidative fibre types in this model. This suggests that mitochondria are also decreasing proportionally with fibre size. Decreased mitochondrial content in the diaphragm has also been observed in mice after four weeks of CH [80].

Mitochondria have previously been observed to be more abundant in the diaphragm sub-sarcolemma around capillaries following CH. Fig. 4.2 presents with more intense staining observed at the cell boundaries. This localisation and crowding of mitochondria may interfere with catalytic activity by this method.

It should be noted that there may be differences in mouse and rat diaphragm muscle metabolic adaptation to CH as differences in pressure generation for different ventilatory activities have been observed [280,281].
4.3.2 Metabolic enzyme activities and HIF-1α

Aldolase – a glycolysis enzyme, aconitase – a TCA cycle enzyme, and CK – a phosphagen system enzyme, all present with decreased activities after six weeks of CH. The diaphragm is adopting a strategy that reduces carbohydrate derivative flux in hypoxia. This is not uncommon in other mammalian hypoxia tolerant tissues as is observed in diving, burrowing, hibernating and high altitude mammals [265,282–287]. Decreasing metabolic activity lends to decreasing ROS production by mitochondria. Correlation with cellular homeostasis, functional integrity and understanding how to achieve this strategy of decreased carbohydrate derivative flux will be important for understanding and managing clinical hypoxia [284]. Understanding how the diaphragm muscle can increase work per unit time despite decreased utilisation of carbohydrate will be further important to muscle physiology. As these enzymes undergo redox remodelling (chapter 3), ROS are potentially candidates for this adaptation.

Aconitase is a readily oxidised enzyme with a ferrous catalytic site that facilitates oxidation-induced decreased catalytic activity which is observed after three and six weeks of CH in the diaphragm. With loss of activity, aconitase has been observed to take on a regulatory role in transcription of proteins involved in iron up-take, storage and utilisation in the cell nucleus [288] as well as stabilising mitochondrial DNA [289]. Aldolase expression is HIF responsive although HIF alone is not enough to drive transcription [290]. However, while aldolase activity is decreased after six weeks of CH and HIF-1α content is increased, there is a suggestion that activity may be increasing again with the six week CH group presenting with higher activities compared to the one and three week CH groups. CK is an enzyme that co-localises with the cross-bridge. CK deficient mice lack the ability for burst activity in muscle and graded deficiencies result in graded burst reductions [242,291]. Burst deficiency likely occurs due to an inability to generate a bulk volume of ATP close to the cross-bridge sufficient for widespread and strong contractions. This is perhaps required by the diaphragm muscle to quickly overcome/prevent periods of dyspnoea and may be important in chronic respiratory disease. Loads on this muscle are relatively low with resting-state breathing. Whilst CK activity is significantly decreased after six weeks of CH in the diaphragm muscle, the increased activity of the muscle may keep
creatine phosphate stores depleted to such an extent that CK is relatively inactive and susceptible to oxidation rather than oxidation targeting this enzyme. Although we do not have CK activity measurements for the sternohyoid muscle, given the oxidation observed in chapter 3 it is likely that similar activity changes occur. Correlating decreased burst activity for this muscle would be of physiological significance given that more forceful contractions are required in this muscle to maintain upper airway patency. This would also be important in progression of OSA where upper airway muscle function is perturbed [292,293].

G6PD and LDH are metabolic enzymes located at key substrate flux gating points. Conflicting with our hypothesis of substrate flux into the pentose phosphate pathway, G6PD is significantly decreased after three and six weeks of CH in the diaphragm. Perhaps, the physiological significance of this is to reduce O2 consumption and ROS production at the NADPH oxidase complex although this is speculative as NADPH from the pentose phosphate pathway is utilised in antioxidant defence mechanisms. Six weeks of CH does not increase NADPH dehydrogenase activity in rat diaphragm muscle [22]. Interestingly, G6PD expression can be HIF-regulated although this is slower than other glycolytic enzymes, depends on redox status also and may not be activated in CH [274,294]. LDH expression is also HIF regulated [290] however there is no evidence to suggest activity begins to rise again after three weeks of CH. HIF alone is not sufficient to drive LDH expression and so different transcriptional regulators may be required for aldolase and LDH genes. Just as we observe decreased activities of key metabolic pathway enzymes, the substrate flux gating point enzymes of G6PD and LDH are also decreased suggesting that substrate flux is not diverted elsewhere and metabolic flux itself is decreasing.

However, increased G3PD (another enzyme located at a metabolic junction) activity after one, three, and six weeks of CH along with increased catalase activity (Chapter 3) is suggestive of increased utilisation of fatty acids as metabolic substrate. Certainly, the high altitude pika diaphragm muscle relies more on β-oxidation than does the diaphragm from the sea-level pika [295]. COPD severity is also correlated with β-oxidation enzyme activity [165]. The contribution of increased G3PD activity to the glycerol-3-phosphate shuttle is potentially important as mice lacking G3PD
are unable to maintain normal ATP levels with exercise [296]. Metabolic substrate utilisation by the heart during hypoxia, to maintain ATP synthesis while minimizing cellular damage by ROS, is intricately regulated [256,297] and there may be complex mechanisms involved to maintain diaphragm functional integrity as the diaphragm is also a vital tissue.

A differential muscle response is also observed in terms of metabolic enzyme activities. GAPDH activity is increased in the diaphragm muscle after one week of CH but decreased after three weeks of CH. This is indicative of an early glycolytic shift in the diaphragm but ROS predominates thereafter to decrease activity. The initial glycolytic shift may be stronger in fast fibre respiratory muscles as both GAPDH and LDH activity increase in the sternohyoid muscle after one week but are decreased after three weeks of CH. In contrast, GAPDH activity is significantly increased after one and three weeks of CH in both EDL and soleus muscles. Furthermore, no decrease in LDH activity was detected. These data indicate that limb muscles favour a glycolytic shift with progressive hypoxia compared to the hypometabolism in the respiratory muscles. These differences further highlight the diaphragm muscle as being unique in terms of adaptation to CH.

It is known that idle enzymes are more readily oxidised and this perhaps is an integral part of the cellular ROS buffering capacity so that working enzymes remain free of redox modification (also discussed briefly in chapter 3). It may be that many of these metabolic enzymes are more prone to oxidation when the animal is placed in hypoxic conditions and this may be responsible for the metabolic activity changes observed in respiratory muscles.

In control conditions, HIF-1α content is less in diaphragm muscle compared to sternohyoid, perhaps reflecting its fibre type composition. Despite this, and unlike the lack of change in the sternohyoid, HIF-1α content is increased with six weeks of CH. Differential roles for HIF-1α may be important in muscles of different fibre type compositions and differential regulation of energy homeostasis [7]. Increased ROS are potentially a requirement for increased HIF-1α in the diaphragm given that chronic supplementation with tempol or NAC attenuated the increase in HIF-1α.
content in the diaphragm but did not affect HIF-1α content in the sternohyoid muscle. A role for ROS in regulation of HIF is also suggested in the literature [148,164,275–277]. Furthermore, greater specificity is also required than simply ROS alone as protein oxidation is also observed in the sternohyoid muscle. The role of increased HIF-1α in the diaphragm after six weeks of CH is difficult to discern given that glycolytic enzyme activities are decreased however we have discussed that HIF alone is not sufficient to stimulate expression of several of these enzymes. Regardless, HIF-1α content is increased and likely to play a role in diaphragm adaptation to CH given that it may drive expression of hundreds of potential target genes that contain hypoxia-responsive elements [298]. The HIF-1:HIF-2 ratio may also be important in hypoxic adaptation in the diaphragm [144].

4.3.3 Conclusion
The diaphragm in hypoxia adopts a strategy of decreased carbohydrate derivative flux with increased reliance on fatty acid oxidation that is differential to other muscles - owing to the data from the high altitude pika, adaptation of the COPD diaphragm, the mRNA differences within the respiratory muscles in regard to metabolic substrate utilisation [165,257,295], and the data presented here with global decreased enzyme activities apart from G3PD and catalase (chapter 3). Gluconeogenesis would also be an option given that there is muscle fibre atrophy and thus protein catabolism but carbohydrate pathway metabolic enzymes activities are decreased. Fatty acid metabolism enzyme activities and reaction products need to be measured in respiratory and limb muscles to confirm their role.

The diaphragm is also more active compared to limb muscles and so there is a requirement for processes to facilitate both hypometabolism and functional integrity – muscle atrophy is discussed in the following chapter.

There is an extensive and interesting comparative physiology literature on metabolism in hypoxia-sensitive and hypoxia-tolerant animals, some of which has been discussed already. One group proposes a two-phase response of hypoxia tolerant systems to an oxygen lack. The first is a defence against hypoxia which involves balanced suppression of ATP-demand and ATP-supply pathways to
stabilize adenylate levels while ATP turnover rates greatly decline. ATP demands must also be down-regulated and translational arrest is one such mechanism. Rescue mechanisms then occur if the period of oxygen deprivation is extended by preferentially regulating the expression of several proteins, and hypoxia tolerant cells use significant gene-based metabolic reprogramming. Hypometabolic steady states may be prerequisite for surviving prolonged hypoxia [299].

Protein phosphorylation has been identified as a reversible mechanism for the regulated suppression of metabolism and thermogenesis during mammalian hibernation. Phosphatase subfamilies are differentially augmented in the hibernating squirrel and these augmentations are organ specific [300]. ROS may also be involved in triggering phosphorylation changes [224].

Figure 4.12: Achieving hypoxia tolerance as described by Ramirez et al., (2007). Failures to reduce metabolic activity, prevent cellular injury, and/or maintain functional integrity is detrimental to hypoxia tolerance (53).
Chapter 5: Atrophy in the diaphragm after chronic sustained hypoxia
5.1 Chapter Introduction

Muscle atrophy is a decrease in muscle volume or muscle constituents due to decreased cell volume and/or decreased protein component of the cell. It occurs in catabolic periods that present in various diseases such as COPD, heart failure, and cancer [71,81,83,301–304] to provide amino acids to other organs (muscles contain the majority of the body’s protein). It can occur in conditions where the muscles themselves become stressed and/or deconditioned e.g. denervation, chronic unloading, prolonged bed-rest, hypogravity, mechanical ventilation, starvation, hypoxia, oxidative stress [16,22,46,89,90,94,277,305–309]. Increased contractile activity stimulates protein turnover [310] preventing ‘wear and tear’ of sarcomere proteins. Unfortunately, muscle atrophy lends itself to poor prognosis in pathophysiological states [311]. Thus, pathways controlling muscle mass and protein turnover are being increasingly researched as potential therapeutic avenues [86]. Atrophy of the diaphragm muscle is perhaps more dangerous because it contributes to respiratory failure as is observed in COPD, cachexia, muscular dystrophy, and other myopathies where diaphragm wasting is observed [48,49,152,312,313].

Skeletal muscle mass is controlled by balancing protein synthesis with degradation. Atrophy can therefore present as an inactive process – simply decreased protein synthesis. Of course, protein synthesis and degradation signalling are intrinsically interlinked and will shortly be discussed. Active atrophy processes in muscle include protein degradation by the proteasome complex or by the lysosome. The difference between these active processes is that individual proteins and small complexes are degraded by the proteasome (microphagy) while whole intracellular organelles such as mitochondria may be imported into the lysosome and degraded therein (macrophagy), although microphagy can also occur at the lysosome.

Atrophy is either rapid or slow in onset depending on conditions experienced by the muscle cell. Slow atrophy of muscle is typically associated with disuse or denervation [314] and we might expect slow atrophy in deconditioned limb muscles in animal models of CH. However, atrophy occurs rapidly (appreciably measurable within a few hours) in diaphragm muscles of mechanically ventilated rats [16].
Rapid onset atrophy is typically observed in fasting states, sepsis and diseases such as cancer cachexia where systemic factors such as circulating glucocorticoids or inflammatory factors are likely to play a role [315–320]. Atrophy is also linked with oxidative stress/signalling [90,150,308,320–323]. Interestingly, metabolic suppression also appears to be a common feature of atrophying muscles [320]. Importantly, muscle fibre atrophy in the diaphragm following CH [22,67] will impact upon muscle function. Elucidating the molecular mechanism and signalling of atrophy in the diaphragm following CH will open potential therapeutic avenues to prevent weakness in hypoxia.

Severe hypoxia has been shown to progressively increase chymotrypsin-like proteasome activity, but not lysosome activity, in rat gastrocnemius muscle after three, seven, and fourteen days of hypoxia exposure [94]. These changes occurred alongside progressive muscle fibre atrophy observed by histology and progressive oxidative stress in the muscle. Increased proteasome activity has also been reported in the COPD diaphragm muscle [85] and increased proteasome subunit mRNA expression is common to several atrophy producing states [320]. Furthermore, proteasome activity is redox sensitive – chymotrypsin-like activity in particular has been detected to be very sensitive to ROS and antioxidant treatment [16,150,217,321], and degradation of oxidised proteins does not require ubiquitination, ATP consumption, or regulation by the 19S “gating” subunit [322]. We sought to measure chymotrypsin-like activity of the 20S proteasome in CH respiratory and limb muscles to determine if proteasome activity is increased as an active atrophy process. Furthermore, if this process is redox driven, comparable results to the muscle specific protein oxidation will be observed.

Atrophy signalling is driven by FOXO3a localisation to the nucleus to transcribe atrophy promoting atrogin mRNAs such as Murf-1 and MAFbx. FOXO3a, in hypertrophic conditions, will be prevented from transcribing mRNA in the nucleus by Akt phosphorylation [88] and can also be inhibited by PGC1-α [324] (Fig 5.1). PGC-1α mRNA and protein have been observed to be reduced in diaphragm muscle of mice exposed to four weeks of CH [80]. FOXO3a transcription activity is also associated with increased catalase activity [90,325] (chapter 3). The MyoD family of
transcription factors, inflammatory cytokines, catecholamines, and MAPK proteins are also involved in modulating the atrophy signal by interaction with the Akt pathway or affecting gene transcription in the nucleus [90,315–320,326]. Hypertrophy signalling and protein synthesis is predominantly driven by circulating growth factors binding to the insulin-like growth factor (IGF)-1 receptor with progressive activation of phosphatidylinositol-3-kinase (PI3K), Akt, mTOR and S6RP promoting both protein transcription and translation [327]. However, there are various stimuli which can impact upon increasing translation such as hypoxia, oxidative stress, contractile activity, and free cytoplasmic amino acids to name a few [328]. Bonaldo and Sandri (2013) provide a comprehensive list of the phenotype of transgenic and K/O mice for genes involved in anabolic and catabolic pathways in skeletal muscle [89]. These pathways can further regulate themselves e.g. FOXOs inhibit mTOR complex 1 (mTORC1) formation in times of energy stress and mTORC2 is shown to facilitate Akt activation [329,330]. Akt and its downstream targets constitute a junction for many signalling proteins in both hypertrophy and atrophy regulation in skeletal muscle as well as being an important pathway themselves. Thus, they are potential candidates of therapeutic manipulation in muscle wasting conditions. However, it should be noted that they affect other biological processes and prevention of protein turnover may be, in the long term, detrimental to cell health [331]. Regardless, very little is known about the activation of these pathways in the atrophied diaphragm muscles of mice exposed to CH. Thus, we sought to quantify the content of phospho-Akt, phospho-mTOR, and phospho-FOXO3a in the diaphragm muscle after one, three and six weeks of CH. Furthermore, we quantified phospho-GSK-3β, phospho-p70S6K, and phospho-S6RP which are important downstream targets of Akt and mTOR. Indeed, several of these targets have also been observed to be regulated by hypoxia dominant to Akt [328]. Quantification of these phosphorylated proteins will aid the full delineation of Akt and other signalling proteins involved in CH-induced diaphragm atrophy signalling. We hypothesized that hypoxia would promote increased atrophy signalling and decreased hypertrophy signalling.

MAPKs represent a large family of protein kinases that regulate various cell signalling cascades and stress responses. They are known to strongly interact with
ROS and the Akt signalling cascade, differentially depending on the specific MAPK protein, in regulation of cell growth. The p38 MAPK increases MAFbx mRNA levels in rat soleus unloading experiments via ROS production [90]. ERK1/2 MAPKs phosphorylate TSC2 at a site independent of Akt to promote mTORC1 formation [332]. JNK MAPK is observed to modulate FOXO3a to promote mitophagy [333]. Furthermore, there are strong associations between increased muscle activity and MAPK activation and the pattern of muscle activation lends to differential MAPK signalling. Prolonged activity is known to activate ERK 1/2 and p38 while JNK activation appears to have greater association with peak force generation and mechanical stress [334–336]. Hypoxia has also been shown to differentially activate MAPK proteins although this is in-vitro and ex-vivo in rat neurons. In Section C of this chapter we measure MAPK pathway protein phosphorylation content after six weeks of CH – specifically phospho-ERK 1/2 (Thr202/204, Tyr185/187), phospho-JNK (Thr183, Tyr185) and phospho-p38 (Thr180, Tyr182).

We should note here that studies involving pair feeding and amino acid supplementation had no effect on atrophy and/or atrophy signals measured in CH limb muscles with or without exercise [94,337]. In rats exposed to three weeks of CH, ~60% of muscle mass is lost independent of hypophagia [338].
Figure 5.1: Hypertrophy/atrophy signalling in muscle. A simplified schematic of intracellular signalling proteins involved in controlling muscle mass and protein turnover. Note: specific downstream effects for several of these proteins are not shown as the effect can vary depending upon the sequence and combination of transcription factor activation but some are discussed in the text. Also, positive and negative regulatory activities are not shown, only the fact that a downstream effect occurs. Furthermore, many cross-talk signals between stimuli and proteins described in non-muscle tissues are omitted for simplification of the diagram. This does not mean they are not active in skeletal muscle however. Phosphorylated content of the cytosolic proteins in the black boxes are assessed in this chapter.
5.2 Chapter Results

5.2.1 Section A: Chymotrypsin-like Proteasome Activity

Chymotrypsin-like activity of the 20S proteasome in diaphragm muscle is shown in Fig. 5.2A and sternohyoid and limb muscles in Fig. 5.2B. Activity in diaphragm muscle is unchanged after one week of CH but significantly increased compared to controls after six weeks (26.12 ± 3.25 vs. 29.85 ± 2.78, 28.69 ± 1.41 vs. 39.68 ± 3.627* mean ± SEM AMC substrate generation (pmol/min/mg protein); control vs. CH; diaphragm chymotrypsin-like proteasome activity after one and six weeks of CH respectively; n= 7-8 per group; *p<0.05). Note: Due to muscle tissue sample volume limitations, activity at the three week time point could not be measured.

After six weeks of CH, activity is unchanged in sternohyoid, increased in EDL (p=0.08), and significantly increased in soleus muscle (30.82 ± 1.88 vs. 34.36 ± 1.16, 13.02 ± 2.48 vs. 19.43 ± 2.36, 16.92 ± 2.41 vs. 33.49 ± 5.71* mean ± SEM AMC substrate generation (pmol/min/mg protein); control vs. CH; sternohyoid, EDL, and soleus chymotrypsin-like proteasome activity after six weeks of CH respectively; n= 4-8 per group; *p<0.05). Note: Due to muscle tissue sample volume limitations, activity at the one and three six week time points could not be measured.
Figure 5.2: Chymotrypsin-like proteasome activity in diaphragm, sternohyoid, EDL, and soleus after sustained hypoxia compared to normoxic controls. (A) Diaphragm chymotrypsin-like proteasome activity (mean ± SEM) after one and six weeks of normoxia or sustained hypoxia expressed as pmol AMC substrate generation/min/mg protein; n=7-8 per group; (B) Sternohyoid, EDL, and soleus chymotrypsin-like proteasome activity (mean ± SEM) after six weeks of normoxia or sustained hypoxia expressed as pmol AMC substrate Generation/min/mg protein; n=4-8 per group; *p<0.05, ns=not significant; Student’s t-test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
5.2.2 Section B: Akt signalling cascade
Phospho-mTOR content in diaphragm muscle is shown in Fig. 5.3A and phospho-FOXO3a content in Fig. 5.3B. Phospho-mTOR content is significantly increased after one week of CH and significantly decreased after three weeks of CH. Content is decreased (p=0.06) after six weeks albeit not statistically significant (92.11 ± 16.04 vs. 146.5 ± 13.36*, 169.4 ± 8.72 vs. 37.21 ± 4.28***, 94.78 ± 9.03 vs. 74.04± 6.49 mean ± SEM normalised relative luminescence units; control vs. CH; diaphragm phospho-mTOR content after one, three and six weeks of CH respectively; n= 7-8 per group; *p<0.05, ***p<0.001).

Phospho-FOXO3a changes are also bi-phasic. Phospho-FOXO3a content is increased after one week of CH (p=0.08) but significantly decreased after three and six weeks of CH (325.7 ± 24.91 vs. 393.3 ± 26.68, 373.6 ± 33.78 vs. 287.1 ± 14.4*, 350.4 ± 23.13 vs. 283.4± 6.53* mean ± SEM normalised relative luminescence units; control vs. CH; diaphragm phospho-FOXO3a content after one, three and six weeks of CH respectively; n= 7-8 per group; *p<0.05).

Phospho-Akt content in diaphragm muscle is shown in Fig. 5.4A and phospho-GSK-3β content in Fig. 5.4B. Phospho-Akt content is unchanged after one week of CH and decreased after three (p=0.06) and six (p=0.07) weeks of CH but does not reach statistical significance (22.12 ± 1.69 vs. 25.71 ± 2.76, 21.98 ± 2.6 vs. 16.81 ± 0.69, 18.87 ± 1.08 vs. 16 ± 0.97 mean ± SEM normalised relative luminescence units; control vs. CH; diaphragm phospho-Akt content after one, three and six weeks of CH respectively; n= 7).

Phospho-GSK-3β changes are bi-phasic. Phospho-GSK-3β content is significantly increased after one week of CH but decreased after three (p=0.055) and six weeks (p=0.054) of CH (31.3 ± 1.93 vs. 47.58 ± 3.33**, 43.95 ± 4.68 vs. 32.25 ± 2.917, 26.18 ± 1.74 vs. 32.53 ± 2.422 mean ± SEM normalised relative luminescence units; control vs. CH; diaphragm phospho-GSK-3β content after one, three and six weeks of CH respectively; n= 7 per group; **p<0.01).
**Figure 5.3: Diaphragm phospho-mTOR and phospho-FOXO3a content after one, three and six weeks of sustained hypoxia compared to normoxic controls.**

(A) Diaphragm phospho-mTOR content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised relative luminescence units; n=7-8 per group; (B) Diaphragm phospho-FOXO3a content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised relative luminescence units; n=7-8 per group; *p<0.05, ***p<0.001; Student’s t-test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); RLU = relative luminescence units.
Figure 5.4: Diaphragm phospho-Akt and phospho-GSK-3β content after one, three and six weeks of sustained hypoxia compared to normoxic controls. (A) Diaphragm phospho-Akt content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised relative luminescence units; n=7 per group; (B) Diaphragm phospho-GSK-3β content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised relative luminescence units; n=7 per group; **p<0.01, ns=not significant; Student’s t-test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); RLU = relative luminescence units.
Phospho-p70S6K content in diaphragm muscle is shown in Fig. 5.5A and phospho-S6RP content in Fig. 5.5B. Phospho-p70S6K content is unchanged after one week of CH, significantly decreased after three weeks of CH, and decreased (p=0.08) after six weeks of CH but not significantly (6.85 ± 0.68 vs. 7.76 ± 0.27, 6.81 ± 0.48 vs. 5.2 ± 0.25*, 6.43 ± 0.3 vs. 5.47 ± 0.41 mean ± SEM normalised relative luminescence units; control vs. CH; diaphragm phospho-p70S6K content after one, three and six weeks of CH respectively; n= 7-8 per group, *p<0.05).

Phospho-S6RP changes are also bi-phasic. Phospho-S6RP content is significantly increased after one week of CH but not different from control levels after three and six weeks of CH (54.21 ± 15.58 vs. 131.3 ± 16.07**, 42.1 ± 5.8 vs. 54.03 ± 12.98, 44.39 ± 8.74 vs. 33.23 ± 4.99 mean ± SEM normalised relative luminescence units; control vs. CH; diaphragm phospho-S6RP content after one, three and six weeks of CH respectively; n= 7 per group; **p<0.01).
Figure 5.5: Diaphragm phospho-p70S6K and phospho-S6RP content after one, three and six weeks of sustained hypoxia compared to normoxic controls. (A) Diaphragm phospho-p70S6K content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised relative luminescence units; n=7 per group; (B) Diaphragm phospho-S6RP content (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as normalised relative luminescence units; n=7 per group; *p<0.05, **p<0.001, ns=not significant; Student’s t-test and Mann-Whitney test as appropriate. Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); RLU = relative luminescence units.
5.2.3 Section C: MAP Kinases

As illustrated in Fig. 5.6A, six weeks of CH increased diaphragm phospho-p38 content compared to control and this was significantly attenuated by chronic antioxidant supplementation with either tempol or NAC (2.606 ± 0.26 vs. 3.996 ± 0.56 vs. 2.215 ± 0.3# vs. 2.112 ± 0.3# mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; diaphragm phospho-p38 content after six weeks of CH; n=5-8 per group; One-way ANOVA: p<0.01, #p<0.05 vs. hypoxia Tukey’s multiple comparison test).

Fig. 5.6B illustrates changes in diaphragm phospho-JNK content after six weeks of CH ± chronic antioxidant supplementation. One-way ANOVA reveals the means are trending towards being significantly different while a multiple comparisons test highlights that six weeks of CH + chronic NAC supplementation significantly decreases phospho-JNK content compared to control (73 ± 13.22 vs. 66.27 ± 10.22 vs. 53.05 ± 8.93 vs. 28.38 ± 5.076* mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; diaphragm phospho-JNK content after six weeks of CH; n=5-8 per group; One-way ANOVA: p<0.01, *p<0.05 vs. hypoxia Tukey’s multiple comparison test).

As illustrated in Fig. 5.6C, six weeks of CH ± chronic antioxidant supplementation has no effect on diaphragm phospho-ERK1/2 content (56.4 ± 16.92 vs.63.38 ± 16.08 vs. 51.35 ± 12.16 vs. 39.7 ± 14.92### mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; diaphragm phospho-JNK content after six weeks of CH; n=5-8 per group; One-way ANOVA: ns).
Figure 5.6: Diaphragm phospho-MAPK contents after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Diaphragm phospho-p38 content (mean ± SEM) expressed as normalised relative luminescence units; n=5-8 per group; (B) Diaphragm phospho-JNK content (mean ± SEM) expressed as normalised relative luminescence units; n=5-8 per group; (C) Diaphragm phospho-ERK 1/2 content (mean ± SEM) expressed as normalised relative luminescence units; n=5-8 per group; *p<0.05 vs. control, #p<0.05 vs. hypoxia Tukey’s multiple comparison test. Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); RLU = relative luminescence units.
As illustrated in Fig. 5.7A, six weeks of CH ± chronic antioxidant supplementation has no effect on sternohyoid phospho-p38 content (1.576 ± 0.14 vs. 1.279 ± 0.176 vs. 1.235 ± 0.079 vs. 1.241 ± 0.29 mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; sternohyoid phospho-p38 content after six weeks of CH; n=5-7 per group; One-way ANOVA: ns).

As illustrated in Fig. 5.7B, six weeks of CH significantly decreased sternohyoid phospho-JNK content compared to control and chronic antioxidant supplementation with either tempol or NAC could not prevent this (104.1 ± 14.47 vs. 63.64 ± 9.496* vs. 65.33 ± 6.65* vs. 48.22 ± 4.7** mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; sternohyoid phospho-JNK content after six weeks of CH; n=5-7 per group; One-way ANOVA: p<0.01, *p<0.05 vs. Control and **p<0.01 vs. Control, Tukey’s multiple comparison test).

Fig. 5.7C illustrates changes in sternohyoid phospho-ERK1/2 content after six weeks of CH ± chronic antioxidant supplementation. The Kruskal-Wallis test reveals the means are significantly different with hypoxia decreasing phospho-ERK1/2 compared to control and this was decreased further with antioxidant supplementation. However, Dunn’s multiple comparisons test revealed no significant differences between pairs (17.2 ± 2.95 vs. 12.28 ± 1.94 vs. 9.367 ± 0.956 vs. 8.752 ± 1.832 mean ± SEM normalised relative luminescence units; control vs. hypoxia vs. tempol vs. NAC; sternohyoid muscle phospho-ERK1/2 content after six weeks of CH; n=5-7 per group; Kruskal-Wallis test: p<0.05).
Figure 5.7: Sternohyoid phospho-MAPK contents after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Sternohyoid phospho-p38 content (mean ± SEM) expressed as normalised relative luminescence units; n=5-7 per group; (B) Sternohyoid phospho-JNK content (mean ± SEM expressed as normalised relative luminescence units; n=5-7 per group; (C) Sternohyoid phospho-ERK 1/2 content (mean ± SEM) expressed as normalised relative luminescence units; n=5-7 per group; *p<0.05 vs. control, **p<0.01 vs. hypoxia, Tukey’s or Dunn’s multiple comparison test as appropriate; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1), RLU = relative luminescence units.
5.3 Chapter Discussion

The main findings of this chapter are 1) chymotrypsin-like activity of the 20S proteasome is significantly increased in diaphragm and soleus muscles after six weeks of CH – similar to protein carbonyl content (chapter 3). Furthermore, basal proteasome activity is greatest in sternohyoid>diaphragm>soleus>EDL which is similar to basal protein carbonyl content in these muscles; 2) six weeks of CH decreases phospho-FOXO3a, phospho-mTOR and increases phospho-p38 MAPK content while antioxidants attenuate increased phospho-p38 MAPK content – suggestive that these are signalling proteins driving hypoxia – and ROS – induced atrophy; 3) atrophy signalling is temporally modulated; and 4) atrophy signalling is differential in diaphragm and sternohyoid muscles despite protein oxidation in both muscles (chapter 3).

5.3.1 Chymotrypsin-like Proteasome Activity

Chymotrypsin-like proteasome activity, which is redox sensitive, is increased in the six week CH mouse diaphragm and soleus muscles. Increased proteasome activity may be redox regulated or may be due to increased proteasome complex formation which would occur in turn with increased atrophy signalling (also potentially due to protein oxidation). Interestingly, these changes follow suit with slow oxidative fibre type muscles being prone to CH-induced protein carbonylation. Furthermore, basal proteasome activity measurements in the different muscles follow suit with basal protein carbonyl contents being greatest in sternohyoid>diaphragm>soleus>EDL muscles. Increased chymotrypsin-like proteasome activity will facilitate degradation of oxidatively damaged proteins, turnover of harder-working contractile apparatus proteins, and will contribute to muscle fibre atrophy in the CH animal model.

The higher respiratory muscle chymotrypsin-like proteasome activity in control conditions is perhaps not surprising given the continuous work of breathing but both highest carbonyl content and proteasome activity in the fast fibre type sternohyoid over diaphragm is unexpected. Perhaps sternohyoid proteasome activity is at approximately maximum capacity in C57Bl6/J control conditions and does not increase to a statistically significant extent after six weeks of CH as a result. Indeed,
the mean CH-diaphragm chymotrypsin-like proteasome activity is not much higher than mean activity in control-sternohyoid.

We cannot rule out lysosomal activity changes as mitochondrial degradation is observed in mouse hypoxic diaphragm alongside decreased PGC-1α and increased BNIP-3 [80]. Increased lysosomal activity more than likely does occur in accordance with these observations. PGC-1α has a role in FOXO suppression (although literature is conflicting) while BNIP-3 targets mitochondria for lysosome degradation [80,324,333,339,340].

5.3.2 Akt signalling cascade

Decreased phospho-mTOR and phospho-FOXO3a content after three and six weeks of CH are indicative of placing a brake on protein translation and facilitating the transcription of pro-atrophy genes respectively. Downstream of mTOR, phospho-p70S6K follows suit and is decreased after three and six weeks. Downstream of p70S6K, phospho-S6RP is not different from controls, suggestive of phosphorylation from alternate pathways that maintains basal translational activity. S6RP can be phosphorylated by the p70 and p90 ribosomal S6 protein kinases, casein kinase 1, and signals from MAPK pathways, as well as dephosphorylated by the protein phosphatase 1 (PP1) [91,341]. Of course, changes in phospho-p70S6K and phospho-S6RP need not necessarily be measurable together with such temporal precision e.g. decreases in p70S6K occur in the mechanically ventilated diaphragm before decreases in protein synthesis are observed [16].

Akt is a modulator of both hypertrophy and atrophy depending on its phosphorylation state. It is upstream of mTOR and FOXO3a and results in phosphorylation of both when activated. Akt phosphorylation occurs when circulating growth factors such as IGF-1 bind to their receptors to initiate a cell growth signalling cascade through the Akt protein. We hypothesized that phospho-Akt content would remain unchanged after CH exposure and that oxidative stress (chapter 3) in the muscle is responsible for the observed muscle fibre atrophy [22,67]. No significant changes in phospho-Akt content were detected; however content changes follow a similar pattern as phospho-mTOR and phospho-FOXO3a.
The decrease in phospho-Akt after three and six weeks of CH may be physiologically relevant over this extended period of time to enhance the signal of decreased protein synthesis and degradation signalling through mTOR and FOXO3a regulation. Basal protein turnover in muscle is very high, potentially more-so in respiratory muscles effecting the hypoxic ventilatory response, and so a large net protein loss over a prolonged period of moderately decreased Akt signalling may occur. However, Akt is unlikely to be the predominant contributor to these processes at the specific time-points given the larger and statistically significant changes observed to the downstream mTOR and FOXO3a in CH. Very severe hypoxia is observed to have no apparent effect on growth factor or catecholamine signalling in rat limb muscle [94].

Akt mediated activation of mTOR is indirect [342,343] through TSC2 and this presents a site of manipulation of mTOR signalling that is downstream of Akt. Indeed, REDD1 and AMPK has been shown to inhibit mTOR signalling through TSC2 [338,344,345]. REDD1 in particular is significantly increased in rat soleus muscle after three weeks of CH [338]. Phospho-Akt content is also significantly decreased in this study. However, there are no data on the effect of REDD1 at an earlier exposure time. Furthermore, REDD1 was not significantly increased in hypoxaemic COPD patient vastus lateralis compared to non-hypoxaemic patients [338].

Further elucidation of the Akt signal strength may be delineated by measuring phosphorylation of its downstream target GSK-3β. GSK-3β is a serine/threonine kinase that was first discovered as a regulatory protein for glycogen synthase but is now recognised as a key regulator of numerous protein functions ranging from metabolic proteins to structural proteins and transcription factors [346]. Indeed, phospho-GSK-3β content is significantly increased after one week of CH unlike phospho-Akt, but follows a similar trend of decreased content as phospho-Akt, similarly not statistically significant, after three and six weeks of CH. There is a regulatory mechanism other than Akt involved at the one week CH time-point.

Like several of these signalling proteins, GSK-3β also regulates feedback control,
over mTOR [347]. Phosphorylation events by GSK-3β, for the most part, result in deactivation of a protein’s activity [319,346–348]. Furthermore, pre-phosphorylation of several GSK-3β substrates is required (priming) before being phosphorylated by GSK-3β – in effect an integration of two signalling pathways. Phosphorylation of GSK-3β can result in increases or decreases in its activity so it is under tight regulation in the cell. GSK-3β phosphorylation at serine 9, the content of which was measured in this study, decreases its activity, thus enhancing activity of its downstream targets.

Specific GSK inhibitors have been observed to attenuate muscle atrophy in a guinea-pig model of LPS-induced atrophy [319]. GSK k/o mice exhibit accelerated sarcopenia of cardiac and skeletal muscle and premature death [347]. In the latter study, GSK deletion induced marked mTOR activation and suppression of atrophy signals. It appears that short-term inhibition of GSK can facilitate muscle growth but long-term inhibition results in reduced degradation of damaged and miss-folded proteins. With regard to this study, increased phospho-GSK-3β measured after one week of CH will be inhibiting muscle growth in and around this period. Phospho-GSK-3β returns to control levels after three and six weeks which may facilitate mTOR signalling thereafter. Of course phospho-mTOR levels have already decreased at these later stages. Potentially, phospho-mTOR activity stimulating growth occurs earlier than the one week time-point and GSK-3β becomes activated to negatively regulate this process. Following this, phospho-p70S6K and phospho-S6RP content are significantly decreased and unchanged respectively after three weeks of CH. Regulation of GSK-3β, and indeed atrophy signalling as a whole, may also play a role in developing the decreased carbohydrate derivative flux strategy observed in the CH-mouse diaphragm [302,320,349,350] as well as that of regulating Ca²⁺ handling proteins [320]. We observed both metabolic enzyme suppression and redox-modulation and expression changes to Ca²⁺ handling proteins in respiratory muscle (chapters 3 and 4). Indeed, just as AMPK feeds information about the energetic state of the cell into the growth signalling pathways [344,345], it is likely that there would be feedback about growth signalling into energetic maintenance. Furthermore, GSK-3β activity is known to regulate HIF-1α expression [351,352].
Regarding regulation of GSK-3β, exposure of muscle to an oxidative stress and insulin significantly reduces GSK-3β phosphorylation at serine9 compared to exposure to insulin alone, almost certainly through decreased Akt phosphorylation which also occurs [353]. Phospho-GSK-3β is increased after one week of CH but returns to control levels by three weeks. A similar finding is observed after four weeks of CH in mouse soleus muscle [354]. Similar to phospho-Akt, GSK-3β is trending toward being significantly decreased after three weeks of CH. However, it is unlikely phospho-Akt is the only upstream effector signal acting upon GSK-3β, especially early in the hypoxia stimulus.

Increased phospho-mTOR, phospho-FOXO3a, and phospho-S6RP after one week of CH suggests there is an early hypertrophic signal, potentially resulting from increased activity of the muscle or an acute ROS stimulus (ROS has been observed to promote adaptation to exercise) [355], that changes in favour of atrophy over time. Both increased synthesis and degradation of proteins in rat limb muscle after two weeks of hypoxia exposure has been reported [94]. Given that phospho-mTOR and phospho-FOXO3a content changes (which are increased after one week and decreased after three weeks) are unlikely to be completely attributable to phospho-Akt and thus may be dephosphorylated at different rates, the potential exists for synthesis and degradation signals to overlap at some point. Some studies present high altitude-induced protein synthesis in human limb muscles but this may be a training effect of ascent to high altitude [356,357]. One study reports that myofibril and sarcoplasmic protein synthesis rates are differentially augmented in human limb muscles. This is not surprising given that contractile proteins are among the most stable in the cell and turnover can take up to ten days. Interestingly, in all of these studies, protein synthesis rates are measured over a relatively short period of time (hours-days). We observe a large and statistically significant increase in phospho-S6RP content after one week of CH. This is likely to be an effect of hypoxia per se given that it is also observed in limb muscles by the aforementioned high altitude studies.

Doucet et al., (2010) [83] present increased mRNA expression of Murf1, MafBx1,
and FOXO1 in COPD patient *vastus lateralis* muscle compared to same patient COPD diaphragm muscles, while phospho-p70S6K protein content was significantly greater in diaphragm compared to *vastus lateralis*. Moreover, these findings suggest that atrophy signalling is either being attenuated in the diaphragm (possibly due to increased activity), potentiated in the limb muscle (possibly due to deconditioning), or both, but only with respect to the other COPD muscle studied. When the diaphragm/vastus ratios for the signalling factors were compared between COPD and control patients, the ratios suggest greater susceptibility to degradation in COPD limb and greater susceptibility to protein synthesis in diaphragm. The implications of this study is that local factors are important and diaphragm contractility and/or limb deconditioning is likely to be important. Interestingly, the PaO$_2$ of patients in this study is not as severe as in other COPD studies. Atrophy of the COPD diaphragm does occur however and literature discrepancies relating to atrophy/hypertrophy signalling in COPD patients have been postulated to be due to the severity of hypoxemia [83, 338]. This is also important for our findings in this study as there are likely to be changes in diaphragm tissue hypoxia overtime due to typical acclimatisation characteristics such as increased red blood cell content and changes in blood flow. However, we have not measured tissue hypoxia in this study.

### 5.3.3 MAP Kinases

Six weeks of CH increases phospho-p38 content in the diaphragm. P38 activity in muscle is strongly associated with promoting atrophy [90, 326]. In fact, one study suggests p38 signalling is required for hypoxia signalling via mitochondrial ROS production [148], which is consistent with our hypothesis of ROS being pivotal in diaphragm adaptation to CH. Phospho-p38 increases are attenuated by antioxidant supplementation further highlighting that this effect of CH is ROS regulated. Both tempol and NAC have been shown to attenuate stress-induced activation of p38 [358]. No change in phospho-p38 content is observed in sternohyoid muscle. The reason for this is unclear however it supports findings from other groups that other local factors are important for driving muscle atrophy [83] in COPD. Furthermore, this may be key as to why ROS in the sternohyoid does not induce degradation of this muscle [22], just as inactive p38 is associated with longevity [359]. ROS
induced activation of p38 occurs through apoptosis-stimulating kinase (ASK1 – a MAP3K) [359–361] activation after release by oxidation of a bound thioredoxin.

Phospho-JNK is decreased by six weeks of CH in diaphragm and more-so in the sternohyoid, with antioxidant treatment (especially NAC) further serving to decrease phospho-JNK content. JNK is also activated by the upstream MAP3K ASK1 [361]; however differential regulation of p38 and JNK occurs at the MAP2K level and is potentially influenced by contractile activity and mechanical stress [362]. Furthermore, ROS activation of phosphatases can differentially regulate MAPK protein activation [363]. Interestingly, both JNK and p38 have been shown to export FOXO proteins from the nucleus which is contradictory to atrophy [364], thus, it is likely to be a specific combination of activated p38, JNK, FOXO, Akt and other signalling kinases and phosphatases that are requisite for the muscle fibre atrophy response. Given the greater decrease of phospho-JNK content in the sternohyoid following CH, basal levels of phospho-JNK are potentially important for CH-induced muscle fibre atrophy in the diaphragm.

Phospho-ERK1/2 content may also be involved in this response too as it is unchanged in the diaphragm after six weeks of CH ± antioxidant supplementation but decreased in the sternohyoid and more-so with antioxidant treatment. ROS induced activation of ERK1/2 in skeletal muscle may require Ca$^{2+}$ signalling [365]. ERK1/2 also activates mTOR through a parallel mechanism to Akt but converging on TSC2 [332] and so ERK1/2 may be more important early in hypoxia adaptation.

The MAPK proteins are typically associated with cell life versus death signalling. We know the diaphragm muscle atrophies with CH and COPD but it does remain functional [22,43,67,70,86,366]. In the clinical setting, patients may present with respiratory disease where hypoxia is featured and although breathing may be difficult and clinical outcome is correlated with inspiratory pressure generating capacity [47], it is only in severe cases that mechanical ventilation is required. Here we present differential MAPK responses in different muscles that will facilitate differential atrophic responses but ultimately the combination of these do not result in apoptosis.
5.3.4 Conclusion

We conclude that hypoxia increases the activity of diaphragm muscle degradation processes and atrophy signalling differential to other respiratory and limb muscles. Oxidative fibre types are potentially more prone to active degradation. Furthermore, atrophy signalling in the diaphragm results in temporal changes in phospho-FOXO3a, phospho-mTOR, and phospho-S6RP content indicating both transcriptional and translational regulation of muscle cell size. Increased p38 MAPK is the likely candidate for driving this response in diaphragm given its redox regulation and association with atrophy signalling. We hypothesize that contractile activity, hypoxia, oxidative stress, and the cellular energetic state contribute to CH-hypertrophy/atrophy changes in diaphragm muscle through the modulatory effects on the signalling proteins presented in this chapter.

Atrophy is often associated with functional and metabolic abnormalities [302,320]. We should note that atrophy may not be entirely maladaptive in periods of hypoxia and oxidative stress. Muscle atrophy will also serve to clear damaged proteins, reduce O₂ diffusion distances, and increase amino acid bioavailability. Less ATP is required for active atrophy than for protein synthesis. However, now that we have a greater appreciation of the molecular mechanisms that underpin muscle fibre atrophy in hypoxia, we can begin to assess different therapeutic avenues e.g. acute GSK-3β inhibition improves muscle growth during sepsis [319]. Given the associations between oxidative stress and active atrophy, and the fact that antioxidant administration can attenuate p38 activation and proteolysis [321], antioxidants may be useful as adjunct therapies in hypoxia-induced respiratory muscle fibre atrophy. Antioxidants are observed to increase phosphorylation of p70S6K [16]. Interestingly, antioxidants have been shown to prevent release of myofibrils from the sarcomere in the mechanically ventilated rat diaphragm, suggesting that ROS promote sarcomeric degradation [321]. The early hypertrophic adaptations are potentially regulated by the antioxidant response in the diaphragm (chapter 3).

Key proteins still need to be turned over in basal conditions, especially in working muscle. Exposure to hypoxia results in a hypoxic ventilatory response that over time
plateaus at a lower rate. Other pathophysiological states resulting in atrophy show some increased gene expression of translational control proteins [320] and we may be observing this early in the hypoxia response of the diaphragm muscle as a protective mechanism.

Given the onset times and direction of fibre type shifts and muscle volume in respiratory and limb muscles from the same COPD patients, local factors appear dominant to systemic factors in driving changes in muscle mass [83,105]. However, glucocorticoids, inflammatory mediators, and catecholamines may also play a role in driving respiratory muscle fibre atrophy in CH and cannot be ruled out without further studies. If they do, perhaps hepatic gluconeogenesis is inappropriately activated in hypoxia as is the case in diabetes [339].

We should note that translation of these data from inbred animal models to the human situation is difficult given that genetic differences account for large variability in muscle performance and structural quality in elite athletes, elderly populations, diseased states and there is also the possibility of sex differences [367].
Chapter 6: Diaphragm function after chronic sustained hypoxia ± antioxidant supplementation
6.1 Chapter Introduction

Diaphragm functional (mal)adaptations in animal models of CH as assessed *ex vivo* include weakness with maintained/improved endurance [22,67], similar to COPD patients [45,56], and are differential in respiratory and in limb muscles [22,67] (discussed in chapter 1). However, there are discrepancies in the literature on diaphragm performance after CH which appear to depend on different *ex vivo* bath temperatures used in the studies [22,26,65]. We have presented in previous chapters protein redox remodelling, a ROS sensitive decreased carbohydrate derivative flux strategy, and evidence of ROS sensitive atrophy signalling and mechanism in mouse diaphragm muscle, differential to other respiratory and limb muscles, after six weeks of CH. While we might associate these molecular adaptations with muscle weakness, they would typically be considered contrary to improved diaphragm endurance. However, diaphragm functional performance following six weeks of CH has only been characterised in rat and not mouse models. It is clear that further assessment of diaphragm function after CH is required. We sought to assess mouse diaphragm and sternohyoid muscle function after six weeks of CH. Given the redox changes observed, we hypothesized that six weeks of CH would be detrimental to mouse diaphragm performance.

Isotonic muscle function, i.e. the ability of the muscle to shorten and at various speeds against loads <100%, is vital to diaphragm function *in vivo*. Isometric contraction, whereby the muscle does not shorten, would fail to produce the requisite thoracic pressure changes for breathing. Only isometric contractile performance of diaphragm muscle bundles following CH has been assessed to date. Furthermore, isotonic and isometric contractions differ greatly at the cellular level such that isotonic performance cannot be predicted from isometric readings [368]. Thus, and more specifically, we sought to assess mouse diaphragm and sternohyoid isotonic muscle function after six weeks of CH. We hypothesized that six weeks of CH would be detrimental to power generating capacity of the diaphragm.

The overall hypothesis for this thesis is that redox remodelling is pivotal for diaphragm muscle adaptation to CH. We have provided evidence of this at a
molecular level. We sought to assess mouse diaphragm and sternohyoid muscle isotonic function after six weeks of CH ± chronic antioxidant supplementation with either tempol or NAC. Antioxidants have been shown to improve skeletal muscle isometric performance and alleviate hypoxia-induced impairment [170,171,369]. Tempol is a membrane permeable superoxide dismutase mimetic while NAC is precursor to the endogenous antioxidant glutathione. As mitochondria are the likely producers of the redox stress, we hypothesize that tempol supplementation will be most beneficial in tempering diaphragm muscle weakness in hypoxia because tempol can permeate into the mitochondria and exert antioxidant effects at ROS source.

The sternohyoid muscle, whose functional performance is studied here in parallel to the diaphragm muscle, presents with similar protein redox changes as diaphragm (chapter 3). However, resultant molecular signalling pathway changes and atrophy mechanism changes (chapter 5, [22]) are differential in these muscles. Thus, antioxidant supplementation may induce changes in sternohyoid muscle performance that are differential to diaphragm. There is a paucity of information concerning sternohyoid muscle function following CH exposure that needs to be addressed, especially given that COPD patients are more prone to upper airway dysfunction and developing OSA [53,54]. Moreover, as is the case with the diaphragm muscle, the existing literature on sternohyoid muscle performance ex vivo after CH is conflicting. Previous work from our laboratory describes no change in rat sternohyoid muscle force or endurance after six weeks of CH and studied at 30°C [22] while another study reports decreased endurance at 25°C [26]. However, isotonic performance assessment may prove differential to isometric conditions given that a significant redox stress is also observed in the sternohyoid following six weeks of CH (chapter 3) and isotonic and isometric contractions differ greatly at the cellular level [368].

Diaphragm performance is reported in section A and sternohyoid in section B. EDL and soleus limb muscles were not studied as the duration of time they would be bathed in Krebs solution ex vivo while diaphragm and sternohyoid function was assessed would result in too great functional deterioration to be of scientific merit.
6.2 Chapter Results

6.2.1 Section A: Diaphragm

Diaphragm muscle twitch contractile kinetics are shown in Fig. 6.1. There are no significant changes in diaphragm TTP (Fig. 6.1A) (15 ± 0.8 vs. 15.8 ± 1.1 vs. 17.2 ± 3.9 vs. 12.9 ± 0.6 mean ± SEM ms; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm TTP after six weeks of CH respectively; n=5-8 per group) or T50 (Fig. 6.1B) (21 ± 0.9 vs. 20.5 ± 2.9 vs. 25.5 ± 4.7 vs. 16.5 ± 2.09 mean ± SEM ms; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm T50 after six weeks of CH respectively; n=5-8 per group) after six weeks of CH ± chronic antioxidant supplementation.

As illustrated in Fig. 6.2, six weeks of CH ± chronic antioxidant supplementation has no effect on peak specific twitch force (Fig. 6.2A) (3.35 ± 0.2 vs. 2.14 ± 0.15 vs. 2.17 ± 0.31 vs. 2.95 ± 0.8 mean ± SEM N/cm²; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm peak specific twitch force after six weeks of CH respectively; n=5-8 per group). However, peak specific tetanic forces (Fig. 6.2B) are significantly different after one-way ANOVA. Tukey’s multiple comparison test reveals that peak specific tetanic force is significantly decreased after CH and CH + tempol but unchanged in the NAC treated group (20.6 ± 0.82 vs. 13.7 ± 1.5** vs. 11.26 ± 0.99*** vs. 22.02 ± 1.3### mean ± SEM N/cm²; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm peak specific tetanic force after six weeks of CH respectively; n=5-8 per group; **p<0.01 vs. control; ***p<0.001 vs. control; ###p<0.001 vs. CH; ###p<0.001 vs. CH + tempol; One-way ANOVA followed by Tukey’s multiple comparison test).
Figure 6.1: Diaphragm isometric twitch kinetics after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Diaphragm time to peak twitch tension (mean ± SEM) expressed as time (ms); n=5-8 per group; (B) Diaphragm twitch half relaxation time (mean ± SEM) expressed as time (ms); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.2: Diaphragm peak specific twitch force and tetanic force after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Diaphragm peak specific twitch force (mean ± SEM) expressed as Force/CSA (N/cm²); n=5-8 per group; (B) Diaphragm peak specific tetanic force (mean ± SEM) expressed as Force/CSA (N/cm²); n=5-8 per group; **p<0.01 vs. Ctrl; ***p<0.001 vs. Ctrl; ###p<0.001 vs. Hypoxia; tttp<0.001 vs. Tempol. Tukey’s multiple comparison test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Diaphragm muscle peak specific shortening velocity is shown in Fig. 6.3A and peak specific shortening in Fig. 6.3B. There are no significant changes in diaphragm peak specific shortening velocity (5.99 ± 0.5 vs. 5.62 ± 0.58 vs. 4.85 ± 0.75 vs. 6.53 ± 0.42 mean ± SEM L_o/s; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm peak specific shortening velocity after six weeks of CH respectively; n=5-8 per group) or diaphragm peak specific shortening (0.53 ± 0.04 vs. 0.46 ± 0.01 vs. 0.41 ± 0.07 vs. 0.51 ± 0.08 mean ± SEM L_o/L_o; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm peak specific shortening after six weeks of CH respectively; n=5-8 per group) after six weeks of CH ± chronic antioxidant supplementation.

As illustrated in Fig. 6.4A, six weeks of CH ± tempol significantly decreases diaphragm peak specific power but no change is observed after CH + NAC treatment (18.91 ± 2.3 vs. 10.2 ± 1.14* vs. 8.74 ± 1.19** vs. 19.02 ± 2.1 tt mean ± SEM Watts/cm^2; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm peak specific power after six weeks of CH respectively; n=5-8 per group; *p<0.05 vs. control; **p<0.01 vs. control; #p<0.05 vs. CH; tt p<0.01 vs. CH + tempol; One-way ANOVA followed by Tukey’s post-hoc test). Peak specific work (Fig. 6.4B) follows a similar pattern as power (2.04 ± 0.33 vs. 1.11 ± 0.1* vs. 0.9 ± 0.12** vs. 1.87 ± 0.2 tt mean ± SEM Watts/cm^2; control vs. CH vs. CH + tempol vs. CH + NAC; diaphragm peak specific power after six weeks of CH respectively; n=5-8 per group; *p<0.05 vs. control; **p<0.01 vs. control; tt p<0.01 vs. CH + tempol; One-way ANOVA followed by Tukey’s multiple comparison test).
Figure 6.3: Diaphragm peak specific shortening velocity and shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Diaphragm peak specific shortening velocity (mean ± SEM) expressed as optimal lengths/Time (L_o/s); n=5-8 per group; (B) Diaphragm peak specific shortening (mean ± SEM) expressed as length/Optimal length (L/L_o); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO_2 = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.4: Diaphragm peak specific power and work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Diaphragm peak specific power (mean ± SEM) expressed as Watts/CSA (cm$^2$); n=5-8 per group; (B) Diaphragm peak specific work (mean ± SEM) expressed as Joules/CSA (cm$^2$); n=5-8 per group; *p<0.05 vs. Ctrl; **p<0.01 vs. Ctrl; #p<0.05 vs. Hypoxia; 'p<0.05 vs. Tempol; "p<0.01 vs. Tempol, Tukey’s multiple comparison test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Diaphragm muscle specific shortening velocity after incremental loads from 0-100% of peak specific force is shown in Fig. 6.5. A two-way ANOVA reveals a significant effect of the treatment (p<0.001) and, as expected, the load (p<0.01), but there is no interaction.

Diaphragm muscle specific shortening after incremental loads from 0-100% of peak specific force is shown in Fig. 6.6. Similar to shortening velocity, two-way ANOVA reveals a significant effect of both treatment (p<0.05) and load (p<0.001) but there is no interaction.

Specific work (Fig. 6.7) and power (Fig. 6.8) after incremental loads from 0-100% of peak specific force however present with significant effects of treatment (p<0.001), load (p<0.001), and treatment-load interaction (p<0.01). Two-way ANOVA followed by Bonferroni’s post-hoc test reveals six weeks of CH ± tempol significantly decreases peak specific work across loads 20-40% but NAC treatment prevents this. Similar results are observed for peak specific power.

Treatment (p<0.001) and time (p<0.001) have a significant effect on diaphragm isotonic fatigue tolerance for all parameters measured – specific shortening velocity (Fig. 6.9), specific shortening (Fig. 6.10), specific work (Fig. 6.11) and specific power (Fig. 6.12) but there is no treatment-time interaction. Bonferroni’s post-hoc test however reveals significant differences in specific work and power between control and CH + tempol over the initial 20s and between CH + tempol and CH + NAC at the 10s time point. In all cases the CH + tempol value is significantly decreased. Significant differences in specific work are also observed from 0-30s in the control vs. CH + tempol groups.
Figure 6.5: Diaphragm shortening velocity-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Diaphragm specific shortening velocity (mean ± SEM) expressed as optimal lengths/Time ($L_o/s$) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.6: Diaphragm shortening-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Diaphragm specific shortening (mean ± SEM) expressed as length/optimal length (L/Lo) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO2 = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-Way ANOVA followed by Bonferroni’s post-hoc test.

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Figure 6.7: Diaphragm work-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Diaphragm specific work (mean ± SEM) expressed as Joules/CSA (cm²) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-way ANOVA followed by Bonferroni’s post-hoc test. All treatments compared at all loads.

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Figure 6.8: Diaphragm power-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Diaphragm specific power (mean ± SEM) expressed as Watts/CSA (cm²) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.9: Diaphragm Fatigue Tolerance: Shortening velocity after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Diaphragm specific shortening velocity (mean ± SEM) expressed as optimal lengths/Time ($L_o$/s) as a function of time (s) with fatiguing stimulation; n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.10: Diaphragm Fatigue Tolerance: Shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Diaphragm specific shortening (mean ± SEM) expressed as length/optimal length (L/L₀) as a function of time (s) with fatiguing stimulation; n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-Way ANOVA followed by Bonferroni’s post-hoc test.

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Figure 6.11: Diaphragm Fatigue Tolerance: Work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Diaphragm specific work (mean ± SEM) expressed as Joules/CVA (cm²) as a function of time (s) with fatiguing stimulation; n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-Way ANOVA followed by Bonferroni’s post-hoc test.

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**Figure 6.12: Diaphragm Fatigue Tolerance: Power after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia.** Diaphragm specific power (mean ± SEM) expressed as Watts/CSA (cm$^2$) as a function of time (s) with fatiguing stimulation; n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
6.2.2 Section B: Sternohyoid

Sternohyoid muscle twitch contractile kinetics are shown in Fig. 6.13. There are no significant changes in sternohyoid TTP (Fig. 6.13A) (9.42 ± 0.31 vs. 10.08 ± 0.37 vs. 10.6 ± 1.12 vs. 10.3 ± 0.4 mean ± SEM ms; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid TTP after six weeks of CH respectively; n=5-7 per group) or T50 (Fig. 6.13B) (11 ± 1.18 vs. 1.67 ± 0.4 vs. 13.9 ± 3.04 vs. 11.7 ± 1.52 mean ± SEM ms; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid T50 after six weeks of CH respectively; n=5-7 per group) after six weeks of CH ± chronic antioxidant supplementation.

As illustrated in Fig. 6.14, six weeks of CH ± chronic antioxidant supplementation has no effect on peak specific twitch force (Fig. 6.14A) (2.5 ± 0.39 vs. 1.56 ± 0.19 vs. 1.88 ± 0.66 vs. 2.99 ± 0.85 mean ± SEM N/cm²; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid peak specific twitch force after six weeks of CH respectively; n=5-7 per group). However, peak specific tetanic forces (Fig. 6.14B) are significantly different after one-way ANOVA. Peak specific tetanic force is decreased after CH and CH + tempol and Tukey’s post-hoc test comparing all means to each other reveals that CH + NAC significantly increases peak specific force compared to CH (12.32 ± 1.49 vs. 7.98 ± 0.58 vs. 11 ± 0.26 vs. 16.03 ± 2.3## mean ± SEM N/cm²; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid peak specific tetanic force after six weeks of CH respectively; n=5-7 per group; ##p<0.01 vs. CH; One-way ANOVA followed by Tukey’s multiple comparison test).
Figure 6.13: Sternohyoid isometric twitch kinetics after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Sternohyoid time to peak twitch tension (mean ± SEM) expressed as time (ms); n=5-7 per group; (B) Sternohyoid twitch half relaxation time (mean ± SEM) expressed as time (ms); n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.14: Sternohyoid peak specific twitch force and tetanic force after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia.

(A) Sternohyoid peak specific twitch force (mean ± SEM) expressed as Force/CSA (N/cm²); n=5-7 per group; (B) Sternohyoid peak specific tetanic force (mean ± SEM) expressed as Force/CSA (N/cm²); n=5-7 per group; ##p<0.01 vs. Hypoxia, Tukey’s multiple comparison test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Sternohyoid muscle peak specific shortening velocity is shown in Fig. 6.15A and peak specific shortening in Fig. 6.15B. There are no significant changes in sternohyoid peak specific shortening velocity (5.4 ± 0.71 vs. 5.19 ± 0.93 vs. 6.11 ± 0.72 vs. 6.05 ± 0.4 mean ± SEM Lₒ/s; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid peak specific shortening velocity after six weeks of CH respectively; n=5-7 per group) or sternohyoid peak specific shortening (0.27 ± 0.03 vs. 0.33 ± 0.05 vs. 0.43 ± 0.04 vs. 0.42 ± 0.07 mean ± SEM L/Lₒ; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid peak specific shortening after six weeks of CH respectively; n=5-7 per group) after six weeks of CH ± chronic antioxidant supplementation.

As illustrated in Fig. 6.16A, There are no significant changes in sternohyoid peak specific power (11.7 ± 1.81 vs. 8.36 ± 1.45 vs. 11.15 ± 1.33 vs. 15.54 ± 2.94 mean ± SEM Watts/cm²; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid peak specific power after six weeks of CH respectively; n=5-7 per group). Peak specific work (Fig. 6.16B) follows a similar pattern as power however one-way ANOVA comparing all groups approaches statistical significance (p=0.08) (0.6 ± 0.09 vs. 0.62 ± 0.08 vs. 0.76 ± 0.09 vs. 0.95 ± 0.11 mean ± SEM Watts/cm²; control vs. CH vs. CH + tempol vs. CH + NAC; sternohyoid peak specific power after six weeks of CH respectively; n=5-7 per group).
Figure 6.15: Sternohyoid peak specific shortening velocity and shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Sternohyoid peak specific shortening velocity (mean ± SEM) expressed as optimal lengths/Time (L₀/s); n=5-7 per group; (B) Sternohyoid peak specific shortening (mean ± SEM) expressed as length/Optimal Length (L/L₀); n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.16: Sternohyoid peak specific power and work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. (A) Sternohyoid peak specific power (mean ± SEM) expressed as Watts/CSA (cm$^2$); n=5-7 per group; (B) Sternohyoid peak specific work (mean ± SEM) expressed as Joules/CSA (cm$^2$); n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Sternohyoid muscle specific shortening velocity after incremental loads from 0-100% of peak specific force is shown in Fig. 6.17. A two-way ANOVA reveals a significant effect of load (p<0.01), but not treatment and there is no interaction.

Sternohyoid muscle specific shortening after incremental loads from 0-100% of peak specific force is shown in Fig. 6.18. Similar to shortening velocity, two-way ANOVA reveals a significant load effect (p<0.001). There is also a significant treatment effect (p<0.001) but there is no interaction. However, Bonferroni’s post-hoc test reveals significantly increased specific shortening in CH + tempol compared to control and CH + NAC compared to control at 0-10% and 0% loads respectively.

Specific work (Fig. 6.19) and power (Fig. 6.20) after incremental loads from 0-100% of peak specific force however present with significant effects of treatment (p<0.001), load (p<0.001), and but no treatment-load interaction. Bonferroni’s post-hoc test reveals increased peak specific work after six weeks of CH + NAC compared to control and CH in loads 25, 40, 60% and 25, 30% respectively.

Shortening velocity, shortening, work and power were measured during the fatigue trial. Time had a significant effect on each parameter studied (p<0.001) as we would expect. Treatment effects were observed for shortening (p<0.001), work (p<0.001), and power (p<0.001), but not shortening velocity. It is clear from Fig. 6.21 that the rate of shortening velocity fatigue is the same in all groups. Differences in sternohyoid rate of shortening fatigue is observed between normoxia and CH + tempol groups as CH + tempol shortening is significantly greater than control at time 0s (p<0.01) but not significantly different at later time points – indicating that the rate of fatigue for tempol with shortening is faster than for normoxia. Similar results are observed for work fatigue whereby CH + NAC work fatigues faster than normoxia and hypoxia alone. CH + NAC power production fatigues faster than hypoxia alone.
Sternohyoid
Shortening Velocity-Load

Figure 6.17: Sternohyoid shortening velocity-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific shortening velocity (mean ± SEM) expressed as optimal lengths/Time (Lo/s) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO2 = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
**Figure 6.18:** Sternohyoid shortening-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific shortening (mean ± SEM) expressed as length/optimal length (L/L₀) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-Way ANOVA followed by Bonferroni’s post-hoc test.

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**Figure 6.19:** Sternohyoid work-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific work (mean ± SEM) expressed as Joules/CSA (cm²) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.20: Sternohyoid power-load after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific power (mean ± SEM) expressed as Watts/CSA (cm²) as a function of load expressed as a percentage of peak force (force/peak force*100); n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 6.21: Sternohyoid Fatigue Tolerance: Shortening velocity after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific shortening velocity (mean ± SEM) expressed as optimal lengths/Time ($L_0/s$) as a function of time (s) with fatiguing stimulation; n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia ($\text{FiO}_2 = 0.1$); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-way ANOVA followed by Bonferroni’s post-hoc test.
All treatments compared at all times.

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Figure 6.22: Sternohyoid Fatigue Tolerance: Shortening after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific shortening (mean ± SEM) expressed as length/optimal length (L/L_o) as a function of time (s) with fatiguing stimulation; n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO_2 = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-Way ANOVA followed by Bonferroni’s post-hoc test.

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Figure 6.23: Sternohyoid Fatigue Tolerance: Work after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific work (mean ± SEM) expressed as Joules/CSA (cm²) as a function of time (s) with fatiguing stimulation; n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Two-Way ANOVA followed by Bonferroni’s post-hoc test.

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Figure 6.24: Sternohyoid Fatigue Tolerance: Power after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Sternohyoid specific power (mean ± SEM) expressed as Watts/CSA (cm$^2$) as a function of time (s) with fatiguing stimulation; n=5-7 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO$_2$ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
6.3 Chapter Discussion

The main findings of this chapter are 1) six weeks of CH is detrimental to diaphragm power generating capacity; 2) tempol supplementation has no mitigating effect on CH-induced diaphragm weakness; 3) NAC supplementation in CH significantly improves diaphragm power generating capacity to an equivalent level to control; 4) both the six weeks of CH group and CH + tempol group improve diaphragm isotonic fatigue tolerance at 33% load compared to control; and 5) both tempol and NAC supplementation in CH improve sternohyoid work-load and power-load relationships compared to CH and control conditions.

6.3.1 Diaphragm

There are no changes in diaphragm contractile kinetics after six weeks of CH and this is consistent with previous studies [22,26,65]. Furthermore, this is suggestive that changes in Ca$^{2+}$ release/re-uptake from the SRs are not occurring in the diaphragm after six weeks of CH and no change is observed to SECRA isoform areal density in rat diaphragm after six weeks of CH [22]. These data suggest that hypoxia does not contribute to the SERCA fast-to-slow isoform transitions which are observed in COPD diaphragm alongside fibre type transitions from fast-to-slow [119], or at least other important factors of the disease are required alongside hypoxia to elicit this adaptation e.g. increased load. The increased force required to overcome increased load in COPD will increase muscle metabolic demand, thus slower relaxation of the muscle to facilitate matching blood perfusion with metabolic demand could be deemed a positive adaptation.

Peak force is decreased after six weeks of CH compared to controls, in agreement with previous findings in animal models of CH [22,65] and of COPD [86]. A similar effect is observed with peak work and power but not peak shortening or shortening velocity. Given that isotonic factors contributing to work and power are unchanged, decreased force generating capacity explains these changes in work and power. Diaphragm weakness is associated with dyspnoea and poor prognosis in respiratory-related diseases with progression resulting in respiratory failure [370] so this effect of CH is clinically relevant. CH in respiratory-related diseases such as COPD is
potentially causal and/or progressive to observed diaphragm muscle weakness [44,56]. There is a role for ROS in effecting these CH-induced performance deteriorations given that NAC supplementation alleviates CH-induced functional weakness. This is potentially the mechanism driving diaphragm weakness in COPD as decreased force is observed in type I and type IIa fibres [44,56] and we have described increased susceptibility of muscles with a higher complement of slow oxidative fibre types to CH-induced protein redox remodelling and degradation.

Curiously, while NAC was inotropic in CH, supplementation with tempol was not. We suggest two reasons why this might be so. Firstly, these antioxidants are administered in the animals’ drinking water during the CH trial and thus must be delivered into muscle to carry out their effects. NAC, which contains a cysteine amino acid and is a precursor to GSH, supplements circulating cysteine and GSH levels. GSH is not a protein and not translated in muscle cells and so GSH/cysteine is transported into tissues from the blood and synthesized in the cell. NAC supplementation increases [GSH] and decreases protein carbonyls in human vastus lateralis muscle [371]. Tempol is a SOD mimetic and SOD enzymes are synthesized in the muscle cells themselves, thus, it is unlikely that muscle cells have transporters to facilitate tempol up-take. Tempol is membrane permeable however, and can pass into muscle down its concentration gradient. Simply, there may be more NAC/GSH/cysteine influx into the diaphragm supplementing antioxidant defences than tempol. However, chronic in vivo tempol supplementation has previously been shown to have inotropic effects on muscle function [189]. Furthermore, in chapter 5 we report that both tempol and NAC supplementation decreased phospho-p38 content to a comparable level as control – indicative that tempol is exerting effects within the diaphragm. Our second hypothesis follows that as tempol is membrane permeable and can permeate into the mitochondria preventing ROS damage at the source, this will also serve to prevent ROS signalling (e.g. turnover of mitochondrial proteins – chapter 3; increased phospho-p38 MAPK – chapter 5), many of which may be important in diaphragm adaptation to CH. NAC may be more limited to supplementing antioxidant defences in the myoplasm and preventing oxidation of sarcomere localised proteins that are important to function directly such as CK, actin, and α-crystallin-B-chain. In a rat model of chronic intermittent hypoxia, both tempol
and NAC supplementation improved diaphragm endurance in CIH but only NAC improved force [189].

As six weeks of CH affects force generating capacity but not shortening velocity, there is likely to be a variable intrinsic to maximum force production, but not maximum shortening velocity [368], that is a target for redox remodelling either directly or downstream of ROS. Certainly oxidation of actin is known to affect acto-myosin interaction (shown in chapter 3) and a similar mechanism may be involved after CH exposure. Metabolic changes will not be driving this; peak performance capacity is determined before fatiguing stimulation is performed. Muscle atrophy is potentially causal because we determine specific force based on CSA of excised tissue and not CSA of individual muscle fibres; force is proportional to FCSA. However, if cross-bridges in parallel are decreased then shortening velocity would also be decreased. Differences in muscle content (e.g. collagen infiltration) are unlikely as previous structural studies on the diaphragm do not present evidence of muscle injury [22,80]. Therefore, the mechanism affecting force but not shortening velocity is likely to be involved in the contractile apparatus. Potentially, redox modifications place cross-bridges in different states of activation under pre-load.

Breathing, for the most part, is against low loads, thus there is a reserve of power available to the diaphragm for physiological function. If, for example, breathing required 8 Watts/cm² (arbitrary value) power generation by the diaphragm, breathing at optimum velocity for power production provides ~57% power generating capacity reserve in control animals but only ~20% in CH animals according to our ex vivo data. Furthermore, producing 8 Watts/cm² can be achieved at a much slower shortening velocity in control than CH animals. This will facilitate greater voluntary control of breathing in controls and be important in development of dyspnoea in respiratory-related disease where hypoxia is featured. The CH diaphragm muscle is clearly at a disadvantage if load increases due to e.g. airway obstruction as observed in COPD. NAC supplementation could ameliorate this weakness in COPD as it does in CH.
Work and power in control diaphragms are significantly greater than CH + tempol diaphragms observed over the initial 30s of the fatigue trial. After 30s, no significant differences are observed suggesting that the rate of isotonic fatigue is faster in the control diaphragm. A fatigue index of the isotonic parameter measured after one minute of fatiguing stimulation as a function of the initial value reveals significantly increased fatigue tolerance in the CH + tempol group compared to control for shortening velocity and power but not shortening or work. Taken together, these data suggest that ROS differentially impact the fatigue tolerance of the isotonic factors of work and power with shortening velocity more susceptible than total shortening. As NAC improves peak force, peak work, and peak power, its fatigue tolerance is also diminished to control levels – in fact CH + NAC fatigue tolerance may be worse than in control later in the fatigue trial.

A potential limitation of the fatigue test in this study is that while initial shortening and shortening velocities for the fatigue tolerance test are quite different across groups, and this accounts for the statistical differences in work and power over the initial 30s, the maximum shortening and shortening velocity at 33% load are not significantly different from each other according to the previous isotonic-load relationships – indicative that significant fatigue has occurred already. This would mean that the CH + tempol group is more prone to fatigue. However, we are still considering fatigue tolerance of the different groups after the same protocol and time spent in the muscle bath *ex vivo*. Furthermore, peak force was re-determined after the step protocol and 100% load was reset accordingly. Thus, regardless of fatigue over the course of the preparation, 33% load was adjusted and accurate to each muscle at the beginning of the fatigue tolerance test. In addition, 33% is representative of the load at which peak power is generated in control animals but not in e.g. CH + tempol treated animals (peak power is generated at ~60% load, Fig.6.8)

Maintained or improved fatigue tolerance of the diaphragm is reported in animal models of CH [22,67] and in COPD [45]. In the *ex vivo* studies, fatiguing stimulation was carried out at 40Hz where isometric force production was not different between control and CH muscles. The 33% load used in this study biases the test in favour of
the weaker CH ± tempol treated groups. Percentage load here is muscle specific, thus shortening against 33% load is a lower absolute load for weaker muscles.

### 6.3.2 Sternohyoid

Similar to diaphragm, CH has no effect on sternohyoid contractile kinetics which is consistent with other studies [22,26] and suggestive that changes in Ca\(^{2+}\) release/re-uptake from the SRs are not occurring in the sternohyoid.

Taking the peak parameter data and isotonic function-load relationships together, CH is detrimental to sternohyoid force, has no effect on shortening velocity, and improves total shortening. As six weeks of CH affects sternohyoid force generating capacity but not shortening velocity, we arrive at a similar scenario as in diaphragm whereby there is likely to be a variable intrinsic to maximum force production, but not maximum shortening velocity [368], that is a target for redox remodelling either directly or downstream of ROS. Differential to diaphragm however, CH improves sternohyoid shortening-load and consequently work-load relationships. Were this effect attenuated by antioxidants, we would credit redox remodelling as the mechanism of adaptation; however antioxidants potentiate CH-induced increased shortening and work. We have discussed increased susceptibility of slow oxidative fibre types to redox remodelling, thus increased shortening capability is potentially an effect specific to fast glycolytic muscles.

Decreased force is contrary to the effects of CH on sternohyoid function reported in rat [22,26], however the temperature used in the latter muscle bath studies were 30°C and 25°C respectively, both lower than the 35°C used in this study. Hypoxia-induced sternohyoid dysfunction is thought to contribute to OSA which is characterised by recurrent airway collapse [292]. Decreased force generating capacity may be detrimental to the bracing function of the muscle potentially increasing susceptibility to airway collapse. Clearly, antioxidant supplementation is a therapeutic strategy to increase sternohyoid work and power.

ROS affect force production in both sternohyoid and diaphragm muscles with consequences for power generating capacity of these muscles. However, differential
muscle isotonic contractile parameter adaptations indicate muscle specific effects of CH on function. This follows suit with differential molecular adaptations presented in previous chapters. However, given that NAC supplementation is beneficial to performance in both muscles, it may be especially useful as an adjunct therapy in the COPD-OSA overlap syndrome.

Fatiguing stimulation had no effect on sternohyoid shortening velocity but treatment effects were observed for shortening, work and power. CH + tempol shortening was significantly greater than control at time zero but not thereafter suggesting fatigue tolerance is decreased in the sternohyoid of the CH + tempol group. CH + NAC work is significantly greater than both CH alone and control at time 10-20s and 0s respectively but not thereafter. This highlights differences in the mechanism of action of tempol and NAC on the sternohyoid in CH. Tempol has greater effect on shortening while NAC has a greater effect on force. In a rat model of chronic intermittent hypoxia, both tempol and NAC supplementation improved diaphragm endurance in CIH but only NAC improved force [189]. A similar effect of NAC is observed for power fatigue tolerance – NAC has no effect on shortening velocity and so increases force generating capacity to increase power. Increased power generating capacity decreases fatigue tolerance. A fatigue index of the work and power measured after one minute of fatiguing stimulation as a function of the initial value reveals decreased fatigue tolerance of the CH + antioxidant groups compared to CH alone. With respect to this, antioxidants may be detrimental to the sternohyoid muscle function in keeping the airway open in CH.

6.3.3 Conclusion
In support of our hypotheses, six weeks of CH is detrimental to mouse diaphragm isotonic performance and this is mitigated by NAC supplementation. Contrary to our hypothesis, tempol had no effect in alleviating CH-induced diaphragm weakness.

Six weeks of CH exposure predominantly affects diaphragm force production which in turn has consequences for the muscle’s ability to perform work and generate power. This is a ROS related phenomenon as it was attenuated by NAC. Some CH-induced ROS may promote positive adaptations in the diaphragm because tempol
had no effect on functional performance in CH. NAC supplementation may be a viable adjunct therapy to improve diaphragm performance in respiratory-related diseases where hypoxia is featured and redox stress occurs. Six weeks of CH also affects sternohyoid force generating capacity and both tempol and NAC supplementation attenuate this. CH-induced ROS in the sternohyoid muscle may be progressive to the development of upper airway dysfunction and OSA that often occurs in COPD patients [53,54].

The strength and site of action of antioxidant supplements is important with regard to improving muscle performance in times of stress as they may block redox signalling that could be important for both positive and negative adaptations rather than negative adaptations alone. Identifying how CH and ROS can be detrimental to diaphragm muscle force but not isotonic shortening or shortening velocity will facilitate greater specificity in identifying and selecting therapeutic strategies to combat respiratory muscle dysfunction.
Chapter 7: Summary & Conclusions
7.1 Summary

Our laboratory has previously described CH-induced structural and functional (mal)adaptations in the diaphragm muscle, similar to COPD patients, and differential in respiratory and limb muscles. Trans-thoracic pressure generating capacity is correlated with survival in COPD so adaptations in the diaphragm following CH are clinically relevant. COPD is predicted to be the 3rd leading cause of death globally and there is no cure. Elucidating the molecular mechanisms that underpin diaphragm adaptations to CH is important for shaping therapeutic strategies. We hypothesized that redox remodelling is pivotal in driving these adaptations for several reasons: 1) CH promotes ROS formation at the mitochondria; 2) mitochondrial remodelling has been described in the diaphragm following CH; respiration rates decreased compared to limb muscles; evidence of mitophagy is observed; and ROS are produced by and contribute to mitochondrial damage; 3) evidence of ROS damage has been observed in skeletal muscle of COPD patients and after high altitude expeditions; 4) contractile activity promotes ROS formation and contractile activity increases in the diaphragm with reflex hyperventilation; and 5) ROS are known to affect muscle at metabolic, structural, and functional levels with adaptations described for each in the diaphragm following CH.

We utilised a mouse model exposed for one, three, and six weeks to CH (FiO₂=0.1) or normoxia for molecular study of the diaphragm. The sternohyoid and limb muscles were studied in parallel as a reference. A second cohort exposed to six weeks of CH ± chronic antioxidant supplementation was used for functional and molecular analysis of the diaphragm.

In chapter three we have highlighted a role for ROS in promoting both bi-phasic and progressive protein redox changes in diaphragm. These changes are differential in respiratory and in limb muscles and occur regardless of increased endogenous antioxidant defences. These data are also indicative that both of the harder-working respiratory muscles have an increased susceptibility to protein redox remodelling as do muscles with a greater complement of slow oxidative fibre types. Following this, we took our investigation of protein redox changes further using a redox proteomics
approach and identified proteins that undergo structural and expression changes following six weeks of CH. It quickly became clear that extensive metabolic enzyme remodelling was occurring, the stress was reaching the level of the cross-bridge, and numerous stress response proteins were affected. These findings prompted us to assess metabolic enzyme activities, atrophy signalling and mechanism, and ultimately function after six weeks of CH + antioxidant supplementation.

Chapter four describes metabolic remodelling in the diaphragm. For the most part, decreased activities of redox remodelled proteins and others at key substrate flux gating points from the glycolysis, TCA cycle and phosphagen metabolic systems are observed. Clearly, a decreased carbohydrate derivative flux strategy is adopted, not an uncommon hypoxia response as is described in the comparative physiology literature, and will serve to put tighter regulation on ATP levels, limit ROS production from mitochondria, and limit cellular acidosis. Some of these changes are bi-phasic, which, taken together with bi-phasic adaptations observed in protein redox remodelling, suggest differential early and late responses to hypoxia at the molecular level. There is also a differential muscle response. Furthermore, we provide some evidence to suggest the diaphragm may increase reliance on fatty acid metabolism which is also reported in the high altitude literature. HIF-1α content is increased in the diaphragm after six weeks of CH and this increase is attenuated by antioxidant supplementation and will contribute to gene expression changes. HIF-1α content remained unchanged in the sternohyoid muscle suggesting a muscle specific activation.

Atrophy signalling changes and a redox sensitive atrophy mechanism are reported in chapter 5. Both active and passive atrophy signalling occur through increased phospho-FOXO3a content and decreased phospho-mTOR content. Moreover, these changes are not driven by Akt, but rather increased phospho-p38 content. P38 MAPK is known to be ROS sensitive and to promote atrophy in times of redox stress. Supplementation with the antioxidants tempol or NAC in CH decreased phospho-p38 content in diaphragm to a comparable level as control. Phosphorylated p38, JNK and ERK1/2 MAPK content changes were differential in diaphragm and sternohyoid muscles and likely underpin the differential muscle fibre atrophy
responses to CH. Bi-phasic changes overtime are further indicative of different early and late adaptive strategies in CH. Redox sensitive chymotrypsin-like activity of the 20S proteasome is also increased in the diaphragm after six weeks of CH as a mechanism of muscle fibre atrophy. Proteasome activity changes after six weeks of CH correlate with protein carbonylation as the muscles with the greater complement of slow oxidative fibres present with the greatest change.

In chapter six we assessed isotonic function of the mouse diaphragm after six weeks of CH ± chronic antioxidant supplementation. Six weeks of CH decreases diaphragm power generating capacity. Decreased force is driving this as shortening velocity was unaffected. Chronic supplementation with tempol had no effect on performance while NAC restored power to a comparable level as control. These data are indicative of redox modification/s at the level of the cross-bridge that affects force production but not shortening velocity in the diaphragm muscle after six weeks of CH. Potentially some ROS are required for redox signalling, and tempol, being a membrane permeable superoxide mimetic, is blocking ROS at source, while NAC is exerting greater effects in the myoplasm of the cell conferring protection to the cross-bridge apparatus whilst permitting some redox signalling.

7.2 Limitations

I appreciate that no research method is without limitations and that the scientific method can only ever be supportive of a hypothesis or prove it false. We hypothesize that redox remodelling is pivotal in driving diaphragm adaptation to CH. Our data on molecular and functional remodelling is in support of this hypothesis. However, no animal model can truly mimic the human scenario; the durations of the hypoxia stimulus are snapshots of the change from control at those particular time-points and oscillations may occur between those time points – just as bi-phasic changes are observed for several of the metabolic signalling proteins studied between control and six weeks of CH; and ex vivo muscle functional performance assessment is potentially different to in vivo performance.
There are also inherent limitations of techniques used in this study: Extracellular matrix (ECM) proteins may be contributing to the redox proteomics data as the ECM is not removed from the muscle. One way around this would be to use single fibres from the muscle however this asks a different question. We are interested in the muscle bundle as a whole which takes away confounding factors such as differences in fibre recruitment during the trial. Ideally all fibres would have to be taken into account and this is not feasible. Given the location and function of the majority of the proteins identified to be remodelled e.g. several metabolic proteins which are present at much higher quantities in muscle than in the ECM, we hypothesize that the contribution of ECM proteins is minimal. A second limitation of this approach is that higher molecular weight proteins are not accounted for. They are known to be difficult to transfer from isoelectric focusing strips into polyacrylamide gel for electrophoresis. Furthermore, if they do transfer across the strip-gel interface, there is a high abundance of high molecular weight proteins in muscle and thus the signal from these is likely to present as saturated and beyond the specification of our measurement tools. An increased amount of saturated protein signals might also mask proteins with very low signals. However, we do use a molecular mass range that incorporates the greatest proportion of different muscle proteins. For isotonic fatigue assessment, we contracted the muscles at 33% load as we and other groups observe 33% to be the approximate load at which peak power occurs. However, the load at which peak power occurs is decreased in the CH + tempol group as we observe from the power-load relationship. Therefore, the fatigue data is not a comparison of peak power fatigue, but simply power fatigue at 33% load. If fatigue data is presented as a percentage of the initial power measured, then the percentage decrease in power highlights the CH + tempol group as being most fatigue tolerant.

Limitations in the conclusions drawn from the evidence: while we suggest that the diaphragm increases reliance on fatty acid oxidation, the evidence to support this, with the exception of catalase, comes from our data on protein activities that do not strictly take part in the beta-oxidation pathway but can support substrate flux through this cascade, as well as from the literature on other models of hypoxia. FOXO3a, unlike the other atrophy signalling proteins assessed, is actually exported from the nucleus and prevented from driving atrophy when it is phosphorylated. Therefore,
measuring a decrease in phospho-FOXO3a content will not strictly be driving atrophy as decreased FOXO3a protein content, phosphorylated or not, could account for this observation. However, coupled with the other changes in the Akt and MAPK cascades, the muscle fibre atrophy phenotype that has been described, and the increased catalase activity, we hypothesize that decreased phospho-FOXO3a is representative of increased non-phosphorylated FOXO3a protein which will drive the fibre atrophy response.

Finally, more protein can be extracted from the diaphragm muscle than the other muscles used in this study. Therefore sternohyoid and limb muscles have not been used as a reference in all experiments described. Furthermore, when sample volumes were running low, some samples were pooled so further experiments could be performed. This in turn decreases the statistical power of some experiments.

7.3 Future Studies

First and foremost, do the molecular mechanisms driving mouse diaphragm adaptation to CH translate to human disease where hypoxia is featured? A molecular investigation of human diaphragm from hypoxic patients and correlation of the magnitude of redox stress with PaO2 would further determine the extent of the translational aspect of this research. Our laboratory is now collaborating with the local university hospital to obtain human biopsy samples to address this.

NAC treatment from the onset of the hypoxic insult proved favourable to diaphragm function but many respiratory-related diseases where hypoxia is featured are detected at later stages of disease. It would be beneficial to know if NAC treatment beginning several weeks after the onset of the hypoxia trial can attenuate or reverse the redox stress and muscle dysfunction.

Future experiments considering muscle fibre atrophy should further record muscle weights to determine if muscle atrophy is commensurate with the observed changes in body weight.
From a scientific perspective, elucidating the mechanism behind CH- and ROS-induced decreased diaphragm force production but not shortening velocity would be interesting. Purification of actin and myosin from mouse diaphragm after CH treatment by chromatography followed by MS analyses to determine sites and types of protein structural remodelling would provide clues as to how this effect materialises.

Another interesting avenue of study would be to determine if these changes can be reversed by a subsequent chronic re-oxygenation protocol following the chronic sustained hypoxia exposure. Given the diaphragm adaptations described in this thesis are redox-sensitive, they are potentially reversible. This is would be of physiological importance to high altitude expeditions in particular where re-oxygenation occurs with descent to sea level.

Lastly, and of great importance for both research and clinical settings where antioxidants are in use, the question remains as to whether chronic tempol supplementation in CH blocks redox signalling events in the diaphragm and in doing so counters potential beneficial antioxidant effects, such as those observed with NAC. If this proves to be the case, then the strength of the antioxidant supplement as well as its target site of action becomes very important in shaping therapeutic strategies that utilise antioxidants.

**7.4 Conclusions & Implications**

We conclude that CH induces protein redox changes in the diaphragm muscle that drive a decreased carbohydrate derivative flux phenotype and promote muscle fibre atrophy. Moreover, these molecular adaptations contribute to decreased diaphragm functional performance after CH, but, importantly, they can be prevented by treatment with the antioxidant NAC but not tempol. These findings indicate that NAC may be a useful adjunct therapy in respiratory-related diseases where hypoxia is featured. However, care must be taken when considering exogenous antioxidants as therapeutic options with regard to their strength and site of action because important redox signalling cascades that result in positive adaptions may be blocked.
e.g. tempol is membrane permeable and will exert stronger antioxidant effects than NAC at the mitochondria. CH-induced adaptations depend on contractile activity, muscle fibre-type composition and duration of exposure and skeletal muscles cannot be considered as a single organ when describing CH-induced changes. Adaptation to CH is temporally modulated and insight drawn from early defensive strategies may not be relevant to longer durations of CH. The redox proteomics approach utilised in this study can be extended to other models of disease characterised by oxidative/reductive stress.
Appendix I

Animal model data for group 1 exposed to one, three and six weeks of CH or normoxia.

Figure 8.1: C57Bl6/J percentage haematocrit after one, three and six weeks of sustained hypoxia compared to normoxic controls. Haematocrit (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed as a percentage of total blood volume; n=8 per group; ***p<0.001; Student’s t-test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Figure 8.2: C57Bl6/J body weights after one, three and six weeks of sustained hypoxia compared to normoxic controls. Body weights (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia expressed in grams (g); n=8 per group; Ctrl = normoxic control; CH = sustained hypoxia (FiO₂ = 0.1).

![Body Weights Graph](image)

Figure 8.3: C57Bl6/J right ventricle/left ventricle (+ septum) weights after one, three and six weeks of sustained hypoxia compared to normoxic controls. Right ventricle/left ventricle (+ septum) weights (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia; n=8 per group; **p<0.01, ***p<0.001, ns=not significant; Student’s t-test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).

![RV/LV Graph](image)
Figure 8.4: C57Bl6/J right ventricle/100g body weight after one, three and six weeks of sustained hypoxia compared to normoxic controls. Right ventricle/(100g) body weight (mean ± SEM) after one, three, and six weeks of normoxia or sustained hypoxia; n=8 per group; ***p<0.001, ns=not significant; Student’s t-test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1).
Animal model data for group 2 exposed six weeks or normoxia or CH ± antioxidant supplementation.

Figure 8.5: C57Bl/6J percentage haematocrit after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Haematocrit (mean ± SEM) expressed as a percentage of total blood volume; n=5-8 per group; ***p<0.001 vs. Ctrl; One-way ANOVA, Tukey’s multiple comparison test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 8.6: C57Bl6/J right ventricle/left ventricle (+ septum) weights after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Right ventricle/left ventricle (+ septum) weights (mean ± SEM); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.

Figure 8.7: C57Bl6/J right ventricle/body weight (100g) after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Right ventricle/body weights (mean ± SEM); n=5-8 per group; *p<0.05 vs. Ctrl; Kruskal-Wallis test, Dunn’s multiple comparison test; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
Figure 8.8: C57Bl6/J body weights after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Body weights (mean ± SEM) expressed in grams (g); n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.

Figure 8.9: C57Bl6/J water consumption after six weeks of sustained hypoxia ± chronic antioxidant supplementation or normoxia. Water consumption (mean ± SEM) expressed in ml/mouse/day; water contained 1mM tempol and 1% NAC for tempol and NAC groups respectively; n=5-8 per group; Ctrl = normoxic control; Hypoxia = sustained hypoxia (FiO₂ = 0.1); Tempol = sustained tempol + sustained hypoxia; NAC = sustained NAC + sustained hypoxia.
List of Publications

- **Conference Abstracts (from this thesis):**


  **Lewis P**, O'Halloran KD (2014) Differential changes in metabolic enzyme activity in murine respiratory and limb muscles after chronic sustained hypoxia. *Young Life Scientists Ireland (Dublin, IRE).* – Oral


  **Lewis P**, Sheehan D, O'Halloran KD (2013) Chronic hypoxia induces differential time-dependent and muscle-specific oxidative changes to the skeletal muscle proteome. *International Hypoxia Symposia (Lake Louise, Canada)* - Poster


Lewis P, McMorrow C, Jones JFX, O'Halloran KD (2011) Chronic hypoxia does not affect rat respiratory muscle fibre oxidative capacity. European Muscle Conference (Berlin, Germany) - Poster

- **Conference Abstracts (not from this thesis):**

Lemaire P, Lewis P, O'Halloran KD (2014) Effects of chronic intermittent hypoxia on rat respiratory muscle metabolic and antioxidant enzyme activities. Royal Academy of Medicine in Ireland Biomedical Sciences Annual Meeting (Dublin, Ireland) - Poster

Williams R, Lewis P, Hogan S, Healy V, O'Halloran KD (2013) NADPH oxidase inhibits rat sternohyoid muscle performance and expression is increased following chronic intermittent hypoxia. Sleep & Breathing (Montreal, Canada) - Oral

Burns D, Lewis P, O'Halloran KD (2013) Xanthine oxidase inhibition improves rat pharyngeal dilator muscle function in vitro. Royal Academy of Medicine in Ireland Biomedical Sciences Annual Meeting (Cork, Ireland) - Poster


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